ASSESSING THE POTENTIAL OF A NOVEL FEED ADDITIVE AND AN UNSATURATED FAT ALONE AND IN COMBINATION TO LOWER METHANE EMISSION FROM CATTLE AND REDUCE THEIR CONTRIBUTION TO CLIMATE CHANGE

by

Megan Lillis Smith

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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DEDICATION

To my loving husband, Manfred E. Marschewski, for all your support and encouragement in furthering my education, without which this process would have been more difficult. I look forward to your continued strength and inspiration in our future endeavors.

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NOMENCLATURE

ADF	acid detergent fiber
AK-PTA	acetate kinase- phosphotransacetylase
AL	atmospheric lifetime
aqH_2	aqueous hydrogen gas
BCFA	branched-chain fatty acids
BES	2-bromoethanesulfonate
BW	body weight
CES	2-chloroethanesulfonate
CFCs	chlorofluorocarbons
CH_3	methane radical
CH_4	methane
CLA	conjugated linoleic acid
CLNA	conjugated linolenic acid
CO	carbon monoxide
CO_2	carbon dioxide
CoA-SH	coenzyme A
CoB-SH	coenzyme B
CODH/ACS	carbon monixide dehydrogenase/acetyl-CoA synthase
CoM-SH	coenzyme M
CoM-S-S-CoB	heterodisulfide of coenzyme M and coenzyme B
CON	control

CP	crude protein
dDMI	digestible dry matter intake
DE	digestible energy
DM	dry matter
DMI	dry matter intake
Ech	energy conserving hydrogenase
$F_{420}H_2$	reduced form coenzyme F420
FA	fatty acids
Fdh	formate dehydrogenase
Fd_{ox}	oxidized form of ferredoxin
Fd_{red}	reduced form of ferredoxin
Fmd	formyl- methanofuran dehydrogenase
Ftr	formyl- methanofuran: tetrahydromethanopterin formyltransferase
GE	gross energy
GEI	gross energy intake
gH_2	gaseous hydrogen gas
GHE	greenhouse effect
GHG	greenhouse gas
GW	global warming
GWP	global warming potential
H^+	hydrogen ion
H_2	dihydrogen
H_2O	water

H_2S	hydrogen sulfide
H_4MPT	tetrahydromethanopterin
HBFCs	hydrobromoflourocarbons
HCFCs	hydrochloroflourocarbons
Hdr	heterodisulfide reductase
HFCs	hydroflourocarbons
Hmd	methylene- tetrahydromethanopterin dehydrogenase
HMG-CoA	hydroxymethylglutaryl
K_m	Michaelis-Menten affinity for substrate constant
Mch	methenyl- tetrahydromethanopterin cyclohydrolase
Mcr	methyl coenzyme-M reductase
Mer	methylene- tetrahydromethanopterin reductase
MES	2-mercaptoethanesulfonate
MFR	methanofuran
MMT CO_2 Eq.	million metric tons of carbon dioxide equivalent
MMT	million metric tons
Mtr	methyl- tetrahydromethanopterin: coenzyme-M methyltransferase
MUFA	monounsaturated fatty acids
N_2O	nitrous oxide
NAD^+	nicotinamide adenine dinucleotide
NE_g	net energy for gain
NE_m	net energy for maintenance
NF_3	nitrogen triflouride

NH_3	ammonia
NMVOCs	non-methane volatile organic compounds
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOP	3-nitrooxypropanol
NOP+OIL	3-nitrooxypropanol + canola oil
NO_x	nitrogen oxides
O_3	ozone
ODS	ozone depleting substances
OH	hydroxyl radical
OIL	canola oil
OM	organic matter
PFCs	periflourocarbons
PI	processing index
ppbv	parts per billion by volume
ppmv	parts per million by volume
PUFA	polyunsaturated fatty acids
RF	radiative forcing
SEM	standard error of measure
SF_6	sulfur hexafluoride
SFA	saturated fatty acids
SO_3^{-2}	sulfite
SO_4^{-2}	sulfate

TDN	total digestible nutrients
TMR	total mixed ration
UFA	unsaturated fatty acids
VFA	volatile fatty acid
ΔG	Gibbs free energy change of a reaction
$\Delta G^{\circ\prime}$	Gibbs standard free energy change of a reaction
ΔG_T	Gibbs free energy change between reactants and products

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ABSTRACT

Methane (**CH_4**) emissions from enteric fermentation of ruminant animals, such as beef and dairy cattle, makes up approximately 2.5% of the total greenhouse gas emissions in the United States and can account from anywhere between 2 and 12% of gross energy intake (**GEI**) (Johnson and Johnson, 1995; IPCC, 2013; EPA, 2015). Therefore, there is an immediate need to decrease CH_4 emission for both environmental as well as economic reasons (Beauchemin et al., 2008). The objective of this study was to assess the potential of 3-nitrooxypropanol, a novel CH_4 inhibitor; and canola oil, a known methane mitigant; alone and in combination on CH_4 emissions, rumen fermentation, and diet digestibility. Eight ruminally cannulated beef heifers (Angus cross, 732 ± 43 kg) were used in a double 4 × 4 Latin square design with four 28-d periods and assigned to one of four dietary treatments. The dietary treatments were: 1) control (**CON**) (no supplementation of 3-nitrooxypropanol or canola oil), 2) canola oil alone (**OIL**) (5% of diet DM), 3) 3-nitrooxypropanol alone (**NOP**) (200 mg/kg of diet dry matter (**DM**); DSM Nutritional Products Ltd., Kaiseraugst, Switzerland), and 4) 3-nitrooxypropanol and canola oil combined (**NOP+OIL**). After a 14-d diet adaption, dry matter intake (**DMI**) was recorded daily. Rumen contents were collected on d 14 and 17 for volatile fatty acid (**VFA**) analysis and protozoal populations. Enteric CH_4 emissions were measured on d 18 to 21 using open circuit chambers. Diet digestibility was measured on d 24 to 27. Methane production was lowered from 26.2 (CON) to 19.6, 17.9, and 12.7 g/kg of DMI, for OIL, NOP, and NOP+OIL, respectively ($P < 0.01$). Total VFA concentrations (m*M*)

were greatest for CON (101.3), similar between OIL (94.8) and NOP (94.8), and lowest for NOP+OIL (88.3) ($P < 0.01$). A decrease in acetate and increase in propionate proportions, and therefore a decrease in the acetate: propionate ratio was also observed with the OIL, NOP, and NOP+OIL treatments compared with CON ($P < 0.01$). The OIL and NOP+OIL treatments had a reduction in protozoa counts and a reduction in DM, organic matter (**OM**), neutral detergent fiber (**NDF**), and acid detergent fiber (**ADF**) digestibilities when compared to CON and NOP (4.43×10^4 vs. 4.24×10^5/ mL rumen fluid; 60.7 vs. 66.8%; 62.0 vs. 68.7%; 47.6 vs. 61.0%; and 46.5 vs. 60.0%, respectively; $P < 0.01$). The results indicated that OIL and NOP are effective means of decreasing CH_4 production, and the combination of both caused the greatest reduction of CH_4 emissions in cattle.

Chapter 1

LITERATURE REVIEW

An Overview of the Greenhouse Effect and Global Warming

The Greenhouse Effect**.** The Greenhouse Effect (**GHE**) is used to describe the relationship of energy exchange between the Sun and the Earth's climate (IPCC, 2007). The Earth's atmosphere is composed of five main layers, including the exosphere and thermosphere (upper atmosphere), the mesosphere and stratosphere (middle atmosphere), and the troposphere (lower atmosphere) (Figure 1). Solar energy enters the Earth's climate at predominantly short wavelengths in the visible or near-visible (ultraviolet) portion of the light spectrum (IPCC, 2007) starting in the upper atmosphere. In reference to Figure 2, approximately one-third to one-half of the solar energy that enters the Earth's upper atmosphere is directly reflected back into space (IPCC, 2007). The remaining one-half to two-thirds of the solar energy is absorbed by the surface of the Earth and the middle and lower atmospheres (IPCC, 2007). In order to counteract the solar radiation, the Earth radiates a similar amount of energy back into space in the form of long wavelengths in the infrared portion of the light spectrum (IPCC, 2007). The difference in absorption of short wavelengths from the Sun and the radiation of long wavelengths from the Earth is due to the difference in temperature, with the Earth being relatively colder than the Sun (IPCC, 2007). The majority of this thermal energy, which is radiated from the land and oceans, is directly absorbed by the atmosphere via clouds and small molecules and is re-radiated back to the Earth, warming the surface of the planet and simulating a natural greenhouse effect (IPCC, 2007). This phenomenon is known as radiative forcing (**RF**) which can be defined as "a change in the balance between incoming solar radiation and outgoing

infrared radiation" (EPA, 2004). Radiative forcing can be both positive (leads to surface warming) or negative (leads to surface cooling) (IPCC, 2013). This natural cycling of solar energy radiation between the Sun and the Earth is essential for the survival of all living organisms on the planet. In fact, without it, the temperature of the Earth's surface would be below 0 °C, the freezing point of water (IPCC, 2007; U.S. Environmental Protection Agency (EPA), 2015).

Global Warming and Greenhouse Gases. The natural capabilities of the Earth's GHE has been intensified due to an accumulation of small molecules in the atmosphere that has caused an increase in the Earth's surface temperature, known as global warming (**GW**), and if it increases significantly, it will make the planet uninhabitable (Sejian and Naqvi, 2012). Although there are a number of small molecules that reside in the atmosphere, not all of them have the capacity to increase the Earth's GHE, and therefore, GW. The atmosphere is composed of predominantly nitrogen (78% of the dry atmosphere) and oxygen (21% of the dry atmosphere), however, neither of these molecules increase the GHE (IPCC, 2007; IPCC 2013). There are numerous more complex molecules that comprise the atmosphere and increase the Earth's GHE and can affect the balance of energy exchange between the atmosphere, space, land, and oceans (EPA, 2015). These molecules are collectively known as greenhouse gases (**GHG**) and include; water (**H_2O**) vapor, carbon dioxide (**CO_2**), CH_4, and nitrous oxide (**N_2O**), and other trace gases. Greenhouse gases come from both natural and man-made (anthropogenic) sources. Natural sources of GHG emissions includes solar and volcanic forcing and other biological systems such as microbial metabolism in wet anaerobic environments (bogs, swamps, peatlands, and other wetland ecosystems), freshwater, wild animals, wildfires, termites, permafrost,

and geological sources (hydrates, seeps, clathates, mud volcanos, and geothermal systems) (IPCC, 2013; NOAA/ ESRL, 2015c). Anthropogenic sources of GHG emissions includes energy, agriculture, industrial processes and product use, waste, and land-use, land-use change, and forestry (discussed in more detail in the next section) (IPCC, 2013; EPA, 2015).

Water vapor. Out of all the gases, H_2O vapor makes the largest contribution to the Earth's GHE (EPA, 2015). Water vapor has an atmospheric lifetime (**AL**) (defined as "the approximate amount of time it would take for the anthropogenic increment to an atmospheric pollutant concentration to return to its natural level (assuming emissions cease) as a result of either being converted to another chemical compound or being taken out of the atmosphere via a sink" (EPA, 2013)) of approximately 10 days (EPA, 2015). It is unlike other GHG and unique in the fact that when it reaches high enough concentrations in the atmosphere, it has the ability to condense into H_2O liquid and can rain out from the atmosphere back to the surface of the Earth (EPA, 2015). It also acts as a feedback mechanism to climate change (IPCC, 2007; EPA, 2015). This is mainly due to two reasons. The first being that the total amount of H_2O vapor that is in the atmosphere at any given time is directly related to the Earth's surface temperature (EPA, 2015). Secondly, the H_2O vapor in the atmosphere can affect the formation of clouds which directly influence the GHE by cooling (reflecting heat from the Sun) or by warming (trapping heat in the atmosphere) the surface of the Earth (EPA, 2015). Essentially, any change in clouds such as type, location, water content, altitude, particle size or shape, and lifetimes can influence their effect on the cooling and warming of the Earth (IPCC, 2013). Therefore, any small increase in GHG could have a greater impact on the GHE due to the

concentration/ condensation and temperature properties of H_2O vapor (EPA, 2015). The global warming potential (**GWP**) is an "index used to translate the level of emissions of various gases into a common measure in order to compare the relative radiative forcing of different gases without directly calculating the changes in atmospheric concentrations" (EPA, 2004). The GWP of GHG is a ratio calculated by using the RF in the hypothetical situation of the release of one kilogram of a GHG to the release of one kilogram of a standard gas (CO_2) over a specified period of time (usually 100 years) (EPA, 2004) and the GWP-weighted emissions can be expressed as:

$$MMT\ CO_2\ Eq. = (kt\ of\ gas)\ \times\ (GWP)\ \times \left(\frac{MMT}{1{,}000\ kt}\right)$$

where **MMT CO_2 Eq.** is million metric tons of carbon dioxide equivalent, kt is kilotons (equivalent to a thousand metric tons), GWP is global warming potential, and **MMT** is million metric tons (EPA, 2015). The GWP of a GHG will vary depending on its concentration in the atmosphere, the atomic lifetime of the gas, and its ability to impact GW (EPA, 2015). The ability of a GHG to impact GW is based on both direct and indirect radiative effects. The radiative effects of GHG are considered direct when the gas itself absorbs radiation and indirect when the gas is either chemically transformed into another GHG or when the gas influences the atomic lifetimes of other GHG (EPA, 2015). However, GWP values are not calculated for small molecules that are involved in complex biological systems or ones that are short lived or unevenly distributed in the atmosphere (such as H_2O vapor and CO_2) (EPA, 2004; EPA, 2015).

Carbon dioxide. Carbon dioxide, although it is the largest contributor to the Earth's GHE after H_2O vapor, the fate of this gas is more variable and based on complex natural geochemical and biological processes (EPA, 2015). Carbon is

naturally cycled between numerous pools including; atmospheric, oceanic, land biota, marine biota, and mineral reserves with the majority of the fluxes being between the atmosphere and oceans and between the atmosphere and terrestrial biota (EPA, 2015). Most of the carbon in the atmosphere exists in the oxidized form as CO_2 and its AL is poorly defined due its cycling variability, but has been suggested its lifetime is anywhere from approximately five to 200 years (EPA, 2015; IPCC, 2001). Carbon dioxide migrates and cycles across different reserves, which makes it difficult to accurately measure (EPA, 2015). Furthermore, CO_2 concentration only slightly increases and some will either decrease slowly over several years or could remain in the atmosphere for thousands of years (EPA, 2015). During the pre-industrial period (defined as the period 1000 – 1750), CO_2 concentrations in the atmosphere were approximately 280 parts per million by volume (**ppmv**) (IPCC, 2001). However, they have increased by 42.5%, leading to CO_2 concentrations of 399 ppmv in 2015 (IPCC, 2001; National Oceanic & Atmospheric Administration/ Earth System Research Laboratory (NOAA/ ESRL), 2015a). According the IPCC and EPA, the main source of anthropogenic CO_2 emissions is the burning of fossil fuels (coal, oil, and gas) with smaller, but still notable, contributions from forest and biomass burning and non-energy production processes (IPCC, 2013; EPA, 2015). Another significant source of CO_2 emissions is due to land-use change, mostly deforestation (IPCC, 2013). However, it is difficult to monitor the CO_2 emissions directly arising from these sources because extensive knowledge such as land area and carbon stored per area, must be known before and after the land-use change (IPCC, 2013).

Methane. Methane is the second most prevalent GHG emitted by anthropogenic sources, with anthropogenic emissions accounting for approximately

70-80% of the total CH_4 emissions (EPA, 2015; Johnson and Johnson, 1995). Emissions of CH_4 have increased exponentially from the pre-industrial period to present day. During the years 1000 – 1750, CH_4 concentrations in the atmosphere were approximately 700 parts per billion by volume (**ppbv**) (IPCC, 2001; EPA, 2015). In the year 2015, CH_4 concentrations had risen to approximately 1,864 ppbv, an increase of 166% (NOAA/ ESRL, 2015b). Although CH_4 has a relatively short AL of only 12 years, it has a GWP value of 25 (IPCC, 2013; EPA, 2015), which was recently updated to a GWP value of 28 to 36 (IPCC, 2014; EPA, 2016). The main sources of CH_4 emissions are from natural biological systems (anaerobic decomposition of organic matter) and from anthropogenic sources (wetland rice cultivation, enteric fermentation in livestock, decomposition of animal and municipal wastes, natural gas and petroleum production and distribution, coal mining, and incomplete fossil fuel combustion) (EPA, 2015). These two general categories are further divided into biogenic, thermogenic, and pyrogenic sources (Neef et al., 2010; IPCC, 2013). Biogenic CH_4 emission sources are due to the anaerobic decomposition of organic matter including natural wetlands, enteric fermentation from ruminants, waste disposal, landfills, rice paddy cultivation, fresh waters, and termites (Neef et al., 2010; IPCC, 2013). Thermogenic CH_4 emission sources are due to the gradual conversion of organic matter to fossil fuels (natural gas, coal, and oil) based on geological time scales (Neef et al., 2010; IPCC, 2013). Pyrogenic CH_4 emission sources are due to the incomplete burning or combustion of organic matter such as biomass and biofuel burning (Neef et al., 2010; IPCC, 2013). There are some natural geological sources that are considered both biogenic and thermogenic, such as oceanic seeps, mud volcanos, and hydrates (Neef et al., 2010; IPCC, 2013). Methane is removed from the

atmosphere mainly by a complex reaction involving the hydroxyl radical (**OH**), and it is ultimately converted to CO_2 or it reacts with chlorine, to a lesser extent, in the marine boundary layer (soil sink) and in stratospheric reactions (EPA, 2015). Due to the complex process involving OH, when CH_4 emissions increase, OH concentrations decrease, which can increase the AL of CH_4 (IPCC, 2013; EPA, 2015).

Nitrous Oxide. Nitrous oxide emissions have increased by 20%, from 270 ppbv during the pre-industrial period to approximately 316 ppbv in the year 2000 (IPCC, 2001; NOAA/ ESRL, 2015b). Although the total N_2O emissions are much lower than CO_2 emissions, N_2O has an atomic lifetime of 114 years and a GWP value of 298, therefore, it is approximately 300 times more powerful at trapping heat in the atmosphere and increasing the GHE when compared to CO_2 (EPA, 2015). The main sources of anthropogenic N_2O emissions includes; agricultural soils (nitrogen-fixing crops and forages), fertilizers (synthetic and manure), feces deposition from livestock, fossil fuel combustion (predominantly mobile combustion), nylon and nitric acid production, waste water treatment, and waste incineration and biomass burning (EPA, 2015).

Trace gases and small molecules. Other trace gases and small molecules that influence the GHE and GW include: ozone (**O_3**), halocarbons, carbon monoxide (**CO**), nitrogen oxides (**NO_x**), non-CH_4 volatile organic compounds (**NMVOCs**), and aerosols (EPA, 2015). Atmospheric O_3 is both positively and negatively, as well as directly and indirectly, related to GW and the GHE. Ozone in the stratosphere is responsible for shielding the Earth from harmful ultraviolet radiation from the Sun, whereas O_3 in the troposphere is responsible for the anthropogenic photochemical "smog" (Figure 1) (EPA, 2015). Stratospheric O_3 has been decreasing over the past

two decades due to ozone depleting substances (**ODS**), such as chlorine- and bromine-containing halocarbons (see next paragraph) (EPA, 2015). This has caused an indirect effect of chlorine and bromine-containing substances and negative RF of O_3 (EPA, 2015). Tropospheric O_3 is categorized as a GHG and has increased by approximately 35% from the pre-industrial period to the year 2000 and is now considered to have the third largest impact on direct RF, behind CO_2 and CH_4, although this is variable depending on the region, latitude, and altitude (IPCC, 2001; EPA, 2015). Tropospheric O_3 is a product of a series of complex chemical reactions involving volatile organic compounds and NO_x in the presence of sunlight; however, the concentrations of O_3 and these pollutants are short lived and spatially variable (IPCC, 2013; EPA, 2015). Therefore, there is no AL or GWP value assigned to O_3.

Halocarbons include anthropogenic gases that contain carbon and halogen (chlorine, fluorine, bromine) that have both direct and indirect RF effects (EPA, 2015). Chlorine-containing halocarbons includes chlorofluorocarbons (**CFCs**), hydrochlorofluorocarbons (**HCFCs**), methyl chloroform, and carbon tetrachloride and bromine-containing halocarbons includes halons, methyl bromide, and hydrobromoflourocarbons (**HBFCs**) (EPA, 2015). Both chlorine- and bromine-containing chemicals are considered to be ODS, as previously stated (EPA, 2015). The use of these substances is also controlled under the Montreal Protocol on Substances that Deplete the Ozone Layer (a treaty signed by 191 countries in the United Nations that called for phasing out the production of ozone-depleting chemicals while implementing ozone-friendly alternatives; EPA, 2012). Fluorine-containing gases; including hydrofluorocarbons (**HFC**), perfluorocarbons (**PFC**), sulfur hexafluoride (**SF_6**) and nitrogen triflouride (**NF_3**) are not categorized as ODS,

and are therefore not regulated under the Montreal Protocol (EPA, 2015). However, they are considered powerful GHGs and their emissions have increased globally over the past fifty years, although their contributions to RF are less than 1% of the total emissions from GHG (IPCC, 2001; IPCC, 2013). Regardless of the fact that they contribute only a small fraction to RF, they have extremely long ALs and therefore, GWPs. The ALs of HFCs, PFCs, SF_6, and NF_3 are: 1-270 years, 2,600-50,000 years, 3,200 years, and 740 years and the GWPs are: 12-14,800, 7,390- 12,200, 22,800, and 17,200, respectively (EPA, 2015). Hydrofluorocarbons are commonly used as replacements for ODS. Perfluorocarbons, SF_6, and NF_3 are released from many industrial processes such as aluminum smelting, semi-conductor manufacturing, electric power transmission and distribution, and magnesium casting (EPA, 2015).

Carbon monoxide has indirect RF effects on the atmosphere by reacting with other molecules (such as OH), which would normally destroy CH_4 and tropospheric O_3 (EPA, 2015). Therefore, it actually increases the levels of these two GHGs. Carbon monoxide is created via the incomplete burning of fossil fuels (EPA, 2015). Under natural processes, CO is normally oxidized to CO_2, therefore it is often short-lived in the atmosphere and highly variable across regions and is not assigned a GWP value (EPA, 2015).

Nitrogen oxides include nitric oxide (**NO**) and nitrate ($\mathbf{NO_2^-}$), both of which have an indirect RF effect on the atmosphere by encouraging the formation of tropospheric O_3 and the formation of nitrate ($\mathbf{NO_3^-}$) aerosol particles in the atmosphere (EPA, 2015). Nitrogen oxides also have positive RF effects by promoting the formation of stratosphere O_3 and negative RF effects by decreasing CH_4 concentrations in the atmosphere (IPCC, 2013; EPA, 2015). Nitrogen oxide emissions

are created from lighting, soil microbial activity, biomass burning, fuel combustion, and from N_2O degradation in the stratosphere (IPCC, 2013; EPA, 2015). Similar to CO, NO_x is short-lived in the atmosphere and highly variable across regions and is therefore not assigned a GWP value (EPA, 2015).

Non-CH_4 volatile organic compounds are substances such as propane, butane, and ethane (EPA, 2015). These compounds combine with NO_x and form tropospheric O_3 and other photochemical oxidants (EPA, 2015). They are emitted as a result of transportation and industrial processes, biomass burning, and non-industrial consumption of organic solvents (EPA, 2015). Like CO and NO_x, NMVOCs are short-lived in the atmosphere and highly variable across regions and are therefore not assigned a GWP value (EPA, 2015).

Aerosols are small particles or liquid droplets in the atmosphere (EPA, 2015). They are created by chemical reactions above the Earth and can be emitted by natural events, such as dust storms and volcanic activity, which produce soil dust, sea salt, biogenic aerosols, sulfates, nitrates, and volcanic aerosols (EPA, 2015). They can also be emitted by anthropogenic events, such as fuel combustion (transportation and coal combustion), cement manufacturing, waste incineration, and biomass burning, which produce industrial dust and carbonaceous aerosol (black carbon or organic carbon) (EPA, 2015). Similar to H_2O vapor, aerosols can be rained out of the atmosphere via precipitation or though other complex processes under dry conditions (EPA, 2015). On the contrary to GHGs, aerosols can have alternative RF effects. For example, the direct RF effects includes the ability to scatter and absorb solar radiation in addition to scattering, absorbing, and emitting terrestrial radiation (EPA, 2015). The indirect RF effects of aerosols includes the ability to increase cloud droplets and ice crystals which

effect the properties of clouds (i.e. formation, precipitation efficiency, and radiative properties) (EPA, 2015). Interestingly, it is believed that aerosols in the atmosphere actually have a net negative RF effect, meaning that they actually have a net cooling effect on the Earth and therefore, they can hide the actual GHE created by increasing emissions of other GHGs (IPCC, 2013; EPA, 2015). Although there is extensive knowledge regarding the cloud-aerosol interactions, there is little known of aerosol effect on RF due to the fact that they have short ALs (days to weeks), various concentrations, sizes, and compositions, and they also vary regionally, spatially, and temporally (which also explains why these small particles are not designated a GWP value) (IPCC, 2013; EPA, 2015).

Sources of Anthropogenic Greenhouse Gas Emissions**.** Out of the top ten GHG producing countries in the world, China, by far, produces the most GHG emissions yearly, with production reaching approximately 10,684 MMT CO_2 Eq. (Figure 3) (World Resources Institute, 2015). The United States produces 5,823 MMT CO_2 Eq. per year and ranks second for total GHG emission production, followed by the European Union (4,123 MMT CO_2 Eq.), India (2,887 MMT CO_2 Eq.), the Russian Federation (2,254 MMT CO_2 Eq.), Indonesia (1,981 MMT CO_2 Eq.), Brazil (1,823 MMT CO_2 Eq.), Japan (1,207 MMT CO_2 Eq.), Canada (856 MMT CO_2 Eq.), and Mexico (749 MMT CO_2 Eq.) (World Resources Institute, 2015). The majority of GHG emissions that were produced in the United States in 2013 came from CO_2 (5,505.20 MMT), CH_4 (636.30 MMT CO_2 Eq.), and N_2O (355.20 MMT CO_2 Eq.), with smaller contributions from HFCs (163.00 MMT CO_2 Eq.), SF_6 (6.90 MMT CO_2 Eq.), PFCs (5.80 MMT CO_2 Eq.), and NF_3 (0.60 MMT CO_2 Eq.) (Figure 4) (IPCC, 2013; EPA, 2015). The Intergovernmental Panel on Climate Change (IPCC) has

summarized all GHG emission sources into five sectors, including; Energy, Agriculture, Industrial Processes and Product Use, Waste Disposal, and Land-use, Land-use Change, and Forestry (IPCC, 2013; EPA, 2015).

Energy. The Energy sector is the largest producer of GHG emissions in the United States (Figure 5). In 2013, this sector was responsible for 5,636,60 MMT CO_2 Eq., which is equivalent to 84.47% of total GHG emissions (IPCC, 2013; EPA, 2015). Most of the GHG emissions from this sector are from fossil fuel combustion (5,157.7 MMT CO_2 Eq.), with minor GHG emissions from natural gas systems (195.2 MMT CO_2 Eq.), non-energy use of fuels (119.8 MMT CO_2 Eq.), coal mining (64.6 MMT CO_2 Eq.), petroleum systems (31.2 MMT CO_2 Eq.), stationary combustion (30.8 MMT CO_2 Eq.), mobile combustion (20.6 MMT CO_2 Eq.), incineration of waste (10.4 MMT CO_2 Eq.), and abandoned underground coal mines (6.2 MMT CO_2 Eq.) (IPCC, 2013; EPA, 2015).

Agriculture. The Agriculture sector is the second largest source of anthropogenic GHG emissions in the United States, contributing 515.70 MMT CO_2 Eq. and representing 7.73% of the total GHG emissions (IPCC, 2013; EPA, 2015). Agricultural GHG emissions sources include agricultural soil management (263.70 MMT CO_2 Eq.), enteric fermentation (164.50 MMT CO_2 Eq.), manure management (78.70 MMT CO_2 Eq.), rice cultivation (8.30 MMT CO_2 Eq.), and field burning of agricultural residues (0.04 MMT CO_2 Eq.), representing 51.14%, 31.90%, 15.26%, 1.61%, and 0.08% of total agricultural GHG emissions, respectively (Figure 6) (IPCC, 2013; EPA, 2015). The two GHGs emitted from the Agriculture sector in 2013 were N_2O and CH_4, comprising 54.52% and 45.48% of the total, respectively (Table 1) (IPCC, 2013; EPA, 2015). Nitrous oxide emissions were mainly from agricultural soil

management (263.70 MMT CO_2 Eq.) with minor emissions from manure management (17.30 MMT CO_2 Eq.) and field burning of agricultural residues (0.10 MMT CO_2 Eq.) (IPCC, 2013; EPA, 2015). On the country, the most prominent CH_4 source from agriculture was enteric fermentation (164.50 MMT CO_2 Eq.) with lesser contributions from manure management (61.40 MMT CO_2 Eq.), rice cultivation (8.30 MMT CO_2 Eq.), and field burning of agricultural residues (0.30 MMT CO_2 Eq.) (IPCC, 2013; EPA, 2015). Methane production from enteric fermentation made up 25.85% of CH_4 emissions and was the single largest contributor to total CH_4 production in the United States in 2013 (IPCC, 2013; EPA, 2015). Ruminant animals, including cattle, sheep, goats, and bison, are the main livestock species that are responsible for emitting large amounts of CH_4 into the atmosphere and increasing GHG emissions, with only minor amounts of CH_4 emissions from monogastric animals. Beef cattle produced 71.14% and dairy cattle produced 25.27% of the CH_4 emissions from enteric fermentation of the Agriculture Sector in 2013, with only 3.58% of the CH_4 emissions from enteric fermentation coming from swine, horses, sheep, goats, American bison, and mules and asses (Figure 7) (IPCC, 2013; EPA, 2015).

Industrial Processes and Product Use. The Industrial Processes and Product Use sector was responsible for 359.10 MMT CO_2 Eq. of GHG emissions, which is equivalent to 5.38% of total GHG emissions produced in 2013 (IPCC, 2013; EPA, 2015). The single largest contributing factor for GHG emissions from this sector is the substitution of ODS (158.6 MMT CO_2 Eq.) with contributions from other industrial sources including iron, steel, metallurgical coke, cement, petrochemical, lime, nitric acid, ammonia, aluminum, carbonates, semiconductor, adipic acid, ferroalloy, titanium dioxide, zinc, phosphoric acid, glass, and lead production/ product

uses (185.00 MMT CO_2 Eq.) and electrical, urea, soda ash, magnesium, CO_2, and silicon carbide production/ processing/ transmission/ distribution/ consumption (15.5 MMT CO_2 Eq.) (IPCC, 2013; EPA, 2015).

Waste Disposal. The Waste Disposal sector contributes 138.30 MMT CO_2 Eq. of GHG, representing 2.07% of the total GHG emissions in the United States in 2013 (IPCC, 2013; EPA, 2015). Landfills are the predominant source of GHG emissions in this sector, followed by wastewater treatment, and composting (114.6, 20.0, and 3.70 MMT CO_2 Eq., respectively) (IPCC, 2013; EPA, 2015).

Land-Use, Land-Use Change, and Forestry. Land-Use, Land-Use Change, and Forestry is the smallest sector in the United States, emitting 23.30 MMT CO_2 Eq. and representing only 0.35% of the total GHG emissions in 2013 (IPCC, 2013; EPA, 2015). The sources of GHG emissions in this sector are forest fires (9.7 MMT CO_2 Eq.), liming of agricultural soils (5.9 MMT CO_2 Eq.), urea fertilization (4.0 MMT CO_2 Eq.), settlement soils (2.4 MMT CO_2 Eq.), peatlands remaining peatlands (0.80 MMT CO_2 Eq.), and forest soils (0.50 MMT CO_2 Eq.) (IPCC, 2013; EPA, 2015).

Enteric Fermentation and Methanogenesis in the Rumen

Enteric fermentation. Ruminant animals and the microorganisms populating their rumens, including bacteria, protozoa, fungi, and Archaea, exist in a synergistic relationship in which both the host and microbial populations mutually benefit. Carbohydrates, including cellulose and starch, are the main energy sources in cattle diets. They are degraded to glucose and metabolized via glycolysis to yield pyruvate (Nelson and Cox, 2005). Pyruvate is metabolized further into a range of products, with the main product being VFA. Volatile fatty acid production in the rumen yields predominantly acetate, propionate, and butyrate with minor production of valerate,

caproate, and branched-chain fatty acids including isobutyrate and isovalerate, additionally yielding CO_2, and H_2. The amount of H_2 from VFA production depends on the dynamics of the rumen and substrate availability/ characteristics. Under normal ruminal conditions, the fermentation of forage will yield predominantly acetate and butyrate and the fermentation of concentrate will yield predominantly propionate (Janssen, 2010; Knapp et al., 2014). Interestingly, in mixed rations, the fermentation profile will favor that similar to forage fermentation, unless the concentrate portion of the ration is greater than 50% (Janssen, 2010). These VFA can provide 50 to 85% of the energy and carbon required for maintenance by the cow, depending on the specific VFA produced and absorbed from the rumen (NRC, 2001). Microbial degradation of proteins and non-protein nitrogen compounds yields amino acids, NH_3, and oligopeptides, which become available for microbial protein synthesis and later lower tract digestion by the cow (Janssen, 2010). Depending on specific rumen conditions, formate, ethanol, lactate, and succinate can also be formed (Janssen, 2010).

The metabolic hydrogen ($\mathbf{H^+}$) produced as a byproduct of fermentation is acted upon by hydrogenase-expressing bacteria and converted to dihydrogen ($\mathbf{H_2}$) within the rumen (Knapp et al., 2014). Dihydrogen, in combination with CO_2 are the main substrates for CH_4 production in the rumen. This process not only acts as the primary H_2 sink by absorbing H_2, but it also re-oxidizes co-factors (nicotinamide adenine dinucleotide; $\mathbf{NAD^+}$) involved in glycolysis, allowing fermentation to continue (Hungate, 1967; Ungerfeld, 2015a). Previous research has demonstrated that the specific proportions of VFA produced and the formation of CH_4 are related to absorption from the rumen, the ability of the rumen utilize aqueous dihydrogen ($\boldsymbol{aq}\mathbf{H_2}$), and the partial pressure of aqH_2 in the rumen (Czerkawski et al., 1972;

Hegarty and Gerdes, 1999; Janssen, 2010). The H_2 balance within the rumen is directly responsible for ruminal fermentation via dynamic control of growth kinetics, thermodynamic control of fermentation pathways, and stoichiometric control of end-production formation (Hungate, 1967; Janssen, 2010). Hydrogen is thus referred to as the "currency" of rumen fermentation (Hegarty and Gerdes, 1999).

The majority of H^+ (91.2%) within the rumen is bound in the form of H_2O and is only available for utilization when liberated from the molecule or utilized in hydrogenation/hydration reactions (Hegarty and Gerdes, 1999). For example, the conversion of glucose to acetate utilizes H_2O, therefore a large portion of the H^+ released during forage fermentation is derived from H_2O and available for the formation of CH_4 or as H_2 (Hegarty and Gerdes, 1999). The remaining H^+ within the rumen is in the form of feed (6.2%), microbial biomass (2.1%), CH_4 (<0.1%), and aqH_2 (<0.1%) (Hegarty and Gerdes, 1999). Although all pools of H^+ influence ruminal fermentation, only aqH_2 is biologically available for utilization into H_2 sinks such as methanogenesis (Janssen, 2010). The concentrations of aqH_2, based on the solubility of H_2 in water and estimated concentrations of gaseous dihydrogen (***g*H₂**) with in the ruminal headspace (0.18%) at 39 ºC, have been estimated to range from 90 to 250 µ*M* (Hegarty and Gerdes, 1999). The concentrations of gH_2 within the headspace and aqH_2 within the rumen fluid were previously deemed to be equivocal (Hegarty and Gerdes, 1999). Summarized in a recent meta-analysis, the concentration aqH_2 was underestimated when based on gH_2 concentrations due to different rates of solubility (Ungerfield et al., 2015b; Wang et al., 2016c). Although aqH_2 equilibrates rapidly with the gH_2 pool in the headspace, the gH_2 pool in the headspace is slow to equilibrate with the aqH_2 pool in the rumen fluid due to the poor solubility of the H_2 in

liquid (Wang et al., 2016c) and therefore, the *aq*H_2 concentrations can not be estimated from the *g*H_2 concentrations (Wang et al., 2016b). This theory was confirmed in sheep (Wang et al., 2016c). In that study, researchers hypothesized that the rumen fluid was supersaturated (defined as "a physical stage at which the concentration of a dissolved gas is above the expected concentration that would result from equilibrium with its gaseous phase") with *aq*H_2 when compared to the *g*H_2 concentration of the headspace, which was later confirmed when they demonstrated that the *aq*H_2 concentrations were greater than the *g*H_2 concentrations in the rumen . They also found that *aq*H_2 concentrations were positively correlated with concentrations of *aq*CH_4 and molar percentage of butyrate and negatively correlated with molar percentage of acetate, although the molar percentage of propionate were non-correlated. This study concluded that not only is it inaccurate to estimated *aq* concentrations of H_2 from the *g*H_2 pool, but when the Gibbs energy change ($\Delta \boldsymbol{G}$) is calculated based on *aq*H2 concentrations, the fermentation pathways producing H_2 (glucose to acetate) had greater ΔG values (less favorable) and the fermentation pathways incorporating H_2 (glucose to propionate and butyrate, reductive acetogenesis, and methanogenesis) had lower ΔG values (more favorable) (Wang et al., 2016c).

Although limited data is currently available, the only accurate way to estimate *aq*H_2 is to measure it directly from the rumen fluid, which ranges from 0.1 to 50 μM (Janssen, 2010). The H_2 concentration in the rumen greatly influences ruminal fermentation by dynamic control of methanogen growth kinetics (H_2 utilization) (Janssen, 2010). The partial pressure of *aq*H_2 in the rumen fluid is influenced by a number of factors including, but not limited to: intake and passage rate, individual

feed ingredients, the chemical composition of feeds, and their ratios included in the diet, and sampling time relative to feeding. The factors affecting *aq*H_2 production, and therefore, CH_4 formation, will be discussed later in detail. Generally speaking, *aq*H_2 in the rumen fluid serves as a source of energy for rumen methanogens. This correlation between an energy source and growth of an organism under normal ruminal conditions can be described by the Monod relationship, which is defined as:

$$\mu = \mu_{max} \times \frac{s}{K_s + s}$$

where μ is the growth rate of the organism, μ_{max} is the maximum possible growth at unlimited concentrations of the energy source under the specific physiochemical conditions, *s* is the concentration of the energy source, and K_s is the Monod constant which describes the energy source at which $\mu = 0.5 \times \mu_{max}$ for that specific microorganism under the specific physiochemical conditions (Figure 8; Janssen, 2010). Based on this concept, when there is a constant input of energy into a system with a microbial population, the microorganisms cannot utilize the energy source fast enough to decrease the concentration of that energy source below the concentrations needed for growth (Janssen, 2010). For instance, according to the equation, when the energy source decreases in concentration, then the growth rate of the organisms also decreases, and vice versa, keeping a steady state of equilibrium between energy source and growth rate. Previous experiments have been summarized by Janssen (2010) and have concluded that in mixed cultures of rumen methanogens and bacteria, the K_s will range from 0 to 50 µ*M*, which is also the concentration range of *aq*H_2 in the rumen fluid. Therefore, small measurable changes in the concentrations of *aq*H2 will interact with the rate of growth of rumen methanogens within the rumen environment (Janssen, 2010).

The H_2 balance within the rumen also influences the thermodynamic control of fermentation pathways, which were briefly discussed earlier (Hungate, 1967; Janssen, 2010). For example, ruminal microorganisms have the ability to change the pathways that they utilize to ferment feeds depending on the concentration of H_2 in the rumen (Janssen, 2010). When the concentration of H_2 is high, the utilization of pathways that produce H_2 become thermodynamically unfavorable, and therefore, the amount of H_2 produced decreases (Janssen, 2010). However, when the concentration of H_2 is low, these H_2-producing pathways become more thermodynamically favorable, and the amount of H_2 produced increases (Janssen, 2010). Although the fermentation of glucose to VFA is dependent on the concentration of the substrate, not all pathways are equally affected by the change in H_2 concentration. This was demonstrated by the change in Gibbs free energy between reactants and products (ΔG_T) over a range of substrate (H_2) concentrations (Figure 9; Janssen, 2010). ΔG_T is more negative, with more energy being released and available for growth of microorganisms, at low concentrations of H_2 compared to higher concentrations of H_2, therefore H_2-producing pathways are more energetically favorable (butyrate + H_2 and acetate + butyrate + H_2) (Janssen, 2010). On the contrary, at high concentrations of H_2, ΔG_T is more positive, therefore, H_2-utilizing pathways are more energetically favorable (acetate + propionate) (Janssen, 2010).

The H_2 balance within the rumen is also directly responsible for stoichiometric control of other end-production (CH_4) formation (Hungate, 1967; Janssen, 2010). The concentration of aqH_2 will affect the amount of CH_4 produced per unit of glucose fermented. For example, under normal ruminal conditions when methanogenesis is

not inhibited, glucose is fermented to end-products (acetate, butyrate, and propionate) via the following equations:

$$Glucose = 0.66acetate + 1.33propionate + 0.66CO_2 + 0.66H_2O + 2H^+$$

$$Glucose = acetate + propionate + CO_2 + H_2 + 2H^+$$

$$Glucose = butyrate + 2CO_2 + 2H_2 + H^+$$

$$Glucose + 0.66H_2O = 0.66acetate + 0.66butyrate + 2CO_2 + 2.66H_2 + 1.33H^+$$

$$Glucose + 2H_2O = 2acetate + 2CO_2 + 4H_2 + 2H^+$$

These equations produce varying amounts of *aq*H_2 and H^+, which can both be used immediately or converted to a bioavailable substrate, which is needed for CH_4 production. Therefore, the amounts of CH_4 produced are as follows for the previously stated equations: 0, 0.25, 0.50, 0.66, and 1.00 mol of CH_4/ mol of glucose (Janssen, 2010).

Methanogenesis in the rumen. As previously stated, enteric fermentation from livestock accounts for approximately 32% of the total GHG emissions from agriculture, with CH_4 comprising approximately 45% of the total emissions from this sector (IPCC, 2013; EPA, 2015). That translates to about 3% of the total anthropogenic GHG emissions in the United States coming from CH_4 emissions from enteric fermentation (IPCC, 2013; EPA, 2015). The majority of CH_4 emissions from enteric fermentation of livestock come specifically from ruminant animals. Although CH_4 is produced in both the upper (rumen) and lower (intestines) gastrointestinal tract, the majority of CH_4 comes from ruminal fermentation, in which anywhere from 250 to 500 L of CH_4 is eructated from the rumen, reticulum, omasum, and abomasum, and expelled via the nose and mouth, and can account for 98% of the total CH_4 emissions from a ruminant animal, with the remaining 2% coming from the rectum (Murray et

al., 1976; Johnson and Johnson, 1995; Stotts, 2016). Not only do CH_4 emissions negatively influence the GHE and GW of the Earth, CH_4 emissions from ruminants can account for two to 12% GEI loss for the animal, depending on animal management (beef or dairy), stage of production (calves, heifer replacements, stockers, lactating or dry cows, or bulls), level of feed intake, and composition of the diet (forage or concentrate, which will be discussed later in more detail) (Johnson and Johnson, 1995; Beauchemin et al., 2009; EPA, 2015).

Methanogenesis in the rumen is a natural biochemical pathway in which substrates are converted to CH_4 via rumen methanogens as a source of energy (Liu and Whitman, 2008). This process acts as a H_2 sink, without it, H_2 would accumulate in the rumen and cause negative feedback which would reduce carbohydrate metabolism, microbial growth, and microbial protein synthesis (Wolin, 1974; McAllister and Newbold, 2008; Knapp et al., 2014). Although there are other alternative H_2 sinks in the rumen, which will be discussed in detail later, the methanogenesis pathway is the most prominent.

Rumen methanogens. The microorganisms that are responsible for the methanogenesis process (also known as biomethylation) in the gastrointestinal tract of ruminants are strict anaerobes and belong to the Archaea domain and the phylum *Euryarchaeota* (Hook et al., 2010; Ferry, 2011). There are seven known orders of methanogenic Archaea, which include Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales, Methanocellales, and the newest addition, Methanomassiliicoccales (previously referred to as "Thermoplasmatales") (Liu and Whitman, 2008; Paul et al., 2012; Lang et al., 2015).

These seven orders are further divided into over 10 families and 31 genera (Liu and Whitman, 2008).

Although many genera of methanogenic Archaea (methanogens) are known, only a few have been identified in the bovine rumen; including *Methanobrevibacter* (62%), *Methanomicrobium* (15%), *Methanoplasmatales* (16%; formally known as Rumen Cluster C (RCC)), with the remaining being from a large group of uncultured rumen Archaea affiliated with *Methanosarcina*, *Methanosphaera*, and *Methanobacterium* (7%; Janssen and Kirs, 2008; Liu and Whitman, 2008; Belanche et al., 2014) (Table 2). Although the total number of methanogenic species in the rumen is unknown (Janssen and Kirs, 2008), it is believed that there are over 900 individual species based on operation taxonomic units (Kim et al., 2011) and that these organisms make up approximately 0.3 to 4.0% of the total rumen microbial biomass (Janssen and Kirs, 2008; Kong et al., 2013; Abecia et al., 2014). Only eight species of methanogens have been identified and cultured from the rumen thus far; including *Methanobrevibacter smithii, Methanbrevibacter ruminantium, Methanobrevibacter thaueri, Methanosphaera stadtmanae, Methanobacterium aarhusense, Methanobacterium formicicum, Methanosarcina barkeri*, and *Methanomicrobium mobile* (Kong et al., 2013).

The first, and most abundant, order of methanogens in the rumen are Methanobacteriales. Members of the Methanobacteriales order includes *Methanobrevibacter (M. smithii*, *M. ruminantium*, and *M. thaueri*), *Methanobacterium* (*M. aarhusense* and *M. formicicum*), and *Methanophaera* (*M. stadtmanae*) (Kong et al., 2013). Most of these organisms use CO_2, H_2, and formate as precursors for CH_4; however, *Methanophaera* can also use methanol as a substrate (Liu and Whitman,

2008). They are usually rod-shaped and 0.6 to 25 µm in size and can form chains which extend approximately 40 µm in length (Jonssen and Kirs, 1979; Liu and Whitman, 2008; Hook et al., 2010). Most species are non-motile (Lui et al., 2008; Liu, 2010a).

The second order of rumen methanogens are Methanomicrobiales. Members of this order include *Methanomicrobium* (*M. mobile*), being similar to species belonging to the Methanobacteriales order, they have the ability to use CO_2, H_2, and formate as precursors for CH_4. Additionally, many species can also use secondary alcohols as substrates for CH_4 production (Liu and Whitman, 2008; Liu, 2010b). Unlike Methanobacteriales, Methanomicrobiales are more diverse in morphology and mobility and can range in shape from cocci to rods (such as *M. mobile*) or sheathed rods and vary in their extent of mobility, with *M. mobile* being quite mobile (Liu and Whitman, 2008; Liu, 2010b).

Methanosarcinales is another order of methanogens found in the rumen. This group contains members from *Methanosarcina* (*M. barkeri*) which are known to have the widest necessity of substrates for CH_4 production, including CO_2 and H_2, but can also disproportionate compounds containing methyl groups such as methylamines and split acetate (Liu and Whitman, 2008; Liu, 2010c). Their morphology is diverse, like members from the Methanomicrobiales order, and can be cocci, pseudosarcinae, or sheathed rods in shape (Liu and Whitman, 2008; Liu, 2010c). However, all members of this order are non-motile (Liu and Whitman, 2008; Liu, 2010c). Additionally, the members of the Methanoscarcinales order are the only methanogens that contain cytochomes (electron carriers that are bound to the membrane and function in the oxidation of methyl groups to CO_2) (Hook et al., 2010). This unique energy

conservation method employs a membrane-bound electron transport chain which is more energy efficient than electron reduction (Lang et al., 2015). Consequently, these methanogens have a growth yield of seven grams per mole of CH_4 (on H_2 and CO_2) and a doubling time of more than 10 h, whereas methanogens without cytochomes have a growth yield of three grams per mole of CH_4 (on H_2 and CO_2) and a doubling time of approximately 1 h (Hook et al., 2010).

The final order of methanogens, which was recently discovered, is the Methanomassiliicoccales order. This order contains members of the *Methanoplasmatales* genus and encompasses the RCC group (Lang et al., 2015). Although little is known about this group, recent research has demonstrated that H_2, methanol, and methylamine are commonly used substrates for CH_4 production and these organisms tend to be irregular cocci in shape (Lang et al., 2015; Nkamga and Drancourt, 2016).

In general, microorganisms of the Archaea domain differ from microorganisms of the Bacteria domain due to the lack of a peptidoglycan membrane in their cell wall (Balch et al., 1979; Hook et al., 2010). In Archaea, this membrane is replaced by a pseudomurein layer in *Methanobrevibacter* and *Methanobacterium*, a heteropolysaccharide layer in *Methanosarcina*, and a protein layer in *Methanomicrobium* (Balch et al., 1979; Hook et al., 2010). All methanogenic Archaea also possess a specific cofactor for the reduction of CO_2, known as F_{420}, which is unique in the fact that it has an absorbance of 420 nm and fluoresces a blue-green color at 470 nm (Hook et al., 2010), which makes a common marker when identifying members of this domain (Ashby et al., 1998). However, although all methanogenic Archaea have this cofactor, it is not unique to this group, as some non-methanogenic

Archaea and groups of *Bacteria* (such as mycobacteria) have also demonstrated procession of this cofactor (Canfield et al., 2005). Archaea also differ from the Bacteria domain in that they use coenzyme M (**CoM-SH**) (from either an internal source in *Methanobacterium* or an external source in *Methanobrevibacter*), which is methylated to produce CH_4 (Hook et al., 2010). Rumen methanogens are associated with the rumen epithelium, rumen fluid, solid feed particles, fungi, as well as with protozoa, both internally and externally (Morgavi et al., 2010; Hook et al., 2010).

Methanogenesis substrates and pathways. There are three predominant substrates that are used by methanogenic Archaea to produce CH_4. These substrates are CO_2-type, methylated C1 compounds, and acetate (Table 3). Different methanogens have varied preferences for these substrates, and depending on the substrate of choice, will influence the methanogenesis pathway that is utilized.

Hydrogenotrophic pathway. The hydogenotrophic pathway (also known as the CO_2-reducing pathway), is the most commonly employed pathway of methanogenesis. As previoulsy stated, the genera of the methanogenic Archaea that employ this pathway are *Methanobrevibacter*, *Methanomicrobium*, *Methanosarcina*, and *Methanoplasmatales*. Carbon dioxide is the major substrate of the hydrogenotrophic pathway, which is predominantly reduced by H_2 as the electron donor, but can also use formate as the electron donor (Figure 10; Liu and Whitman, 2008). When the latter is used, four molecules of formate are oxidized by formate dehydrogenase (**Fdh**), releasing the F_{420} coenzyme and H_2 molecule (**$F_{420}H_2$**), and reduced to one molecule of CO_2 (Liu and Whitman, 2008). It was previously reported that approximatly 18% of the CH_4 produced in the rumen comes from the oxidation of formate (Hungate et al., 1970). In the first step, ferredoxin (**Fd**) is reduced with *aq*H_2 (which serves as the

direct electron donor) via energy conserving hydrogenase (**Ech**), making a reduced form of ferredoxin (**Fd_{red}**). The CO_2 molecule binds to methanofuran (**MFR**) with the help of coenzyme-M (**CoM-SH**) and is then reduced to formyl-MFR by Fd_{red} via formyl-MFR dehydrogenase (**Fmd**). Formyl-MFR is acted upon by tetrahydromethanopterin (**H_4MPT**) and is converted to 5-formyl-H_4MPT via formyl-MFR:H_4MPT formyltransferase (**Ftr**). 5-formyl-H_4MPT is dehydrated by methenyl-H_4MPT cyclohydrolase (**Mch**), releasing H_2O, and forming 5,10-methenyl-H_4MPT. A second molecule of *aq*H_2 hydrogenates F_{420} to $F_{420}H_2$ via F_{420}-reducing hydrogenase, which then acts on 5,10-methenyl-H_4MPT and is catalyzed by methylene-H_4MPT dehydrogenase (**Hmd**) to form 5,10-methenyl-H_4MPT. This intermediate molecule is then reduced again with $F_{420}H_2$ by methylene-H_4MPT reductase (**Mer**), producing 5-methyl-H_4MPT. 5-methyl-H_4MPT is acted upon by CoM-SH, forming methyl-CoM via methyl-H_4MPT:CoM-SH methyltransferase (**Mtr**). In the final step of hydrogenotrophic methanogenesis, methyl-CoM is converted to CH_4 by one of the key enzymes in the methanogenesis pathway, methyl-CoM reductase (**Mcr**). During this conversion, coenzyme-B (**CoB-SH**) acts as the electron donor, forming a heterosulfide bond with CoM (**CoM-S-S-CoB**). This complex is then reduced with $2H^+$ via heterodisulfide reductase (**Hdr**), which regenerates CoM-SH and CoB-SH, and produces CH_4. Energetically and thermodynamically speaking, the reduction of CO_2 to formyl-MFR is an endergonic reaction that is driven by an ion gradient by Ech (Liu and Whitman, 2008). However, net energy is conserved during this pathway via the conversion of the methyl group from 5-methyl-H_4MPT to methyl-CoM via Mtr and the reduction of CoM-S-S-CoB complex to CoM-SH and CoB-SH via Hdr are both exergonic reactions (Liu and Whitman, 2008). Overall, under standard conditions, the

Gibbs standard free energy change (**ΔG′°**) for the CO_2-reducing type of reactions involved in the hydrogenotrophic pathway range from -196 to -37 kJ/mol, and with the Michaelis-Menten affinity for substrate constant (**K_m**), being around 8 μ*M*, this allows the reduction of CO_2 to CH_4 to proceed forward (Table 3; Zinder, 1993; Hedderich and Whitman, 2006; Liu and Whitman, 2008).

Methylotrophic pathway. The methylotrophic pathway, is the second most commonly employed pathway of methanogenesis. As previoulsy stated, the genera of the methanogenic Archaea that employ this pathway are *Methanomicrobium*, *Methanosarcina*, *Methanophaera,* and *Methanoplasmatales*. Methylated C1 compounds, such as methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulfides (methanethiol and dimethylsulfide), are the major substrates of the methylotrophic pathway (Figure 11; Liu and Whitman, 2008). The methlytrophic pathway is similar to the hydrogenotrophic pathway in which the conversion of substrate to CH_4 employes the same intermediate molecules, cofactors, and enzymes. However, instead of the substrate being involved in the first step of methanogenesis, when methylated C1 compounds are used, they enter the pathway at the last step of methanogenesis, just prior to the formation of CH_4. When methylated C1 compounds enter the pathway, they are combined with CoM via methyltransferase, and form methyl-CoM. From this point, the methyl-CoM molecule can proceed in one of either two directions. The most prominent pathway is the conversion of methyl-CoM to CH_4 via similar steps as in the hydrogenotrophic pathway. However, in the methylotrophic pathway, when CoM-SH is regenerated from the CoM-S-S-CoB complex, instead of being used in the conversion of 5-methyl-H_4MPT to methyl-CoM, it is transferred to the next

methylated C1 compound and is used for its conversion to methyl-CoM. In the second, and less prominent pathway, methyl-CoM is converted to CO_2 via the reverse reactions of the hydrogenotrophic pathway. The conversion of methyl-CoM to CH_4 occurs three quarters of the time, whereas the conversion of methyl-CoM to CO_2 via the reverse reactions of the hydrogenotrophic pathway, occurs only one quarter of the time (Liu and Whitman, 2008). Therefore, for every molecule of CO_2 produced, three methyl groups are reduced to CH_4 (Liu and Whitman, 2008). This reaction is termed disproportional since a portion of the substrate is oxidized and subsequently used to reduce the remaining substrate (Liu and Whitman, 2008). Overall, under standard conditions, the $\Delta G'^{\circ}$ for the methyl compound-type of reactions involved in the methylotrophic pathway range from -113 to -49 kJ/mol, which allows the reduction of methylated C1 compounds to CH_4 to proceed forward (Table 3; Zinder, 1993; Hedderich and Whitman, 2006; Liu and Whitman, 2008).

Aceticlastic pathway. The aceticlastic pathway, is the third and least commonly employed pathway of methanogenesis in the rumen. The genus of the methanogenic Archaea that use this pathway are *Methanosarcina,* although they generally prefer methyl-type compounds over acetate for CH_4 production. Acetate is the only substrate used in this pathway and is acted upon by coenzyme-A (**CoA-SH**) and is converted to methylated acetyl-CoA (**CH_3-CO-S-CoA**) via acetate kinase-phosphotransacetylase (**AK-PTA**); (Figure 12; Liu and Whitman, 2008). This intermediate molecule is split via carbon monoxide dehydrogenase/acetyl-CoA synthase (**CODH/ACS**), to yield a molecule of acetyl-CoA and a methyl radical (**CH_3**). Acetyl-CoA is oxidized by Fd to produce a molecule of CO_2 and regenerate CoA-SH. During this oxidation, reduced Fd is dehydrgenated via Ech to H_2. The

methyl radical is combined with H_4MPT to produce 5-methyl-H_4MPT, which follows similar steps as previously described in the hydrogenotrophic pathway, and is reduced into CH_4. Overall, under standard conditions, the $\Delta G'^{\circ}$ for the acetate reaction involved in the aceticlastic pathway is -33 kJ/mol, which allows the reduction of acetate to CH_4 to proceed forward (Table 3; Zinder, 1993; Hedderich and Whitman, 2006; Liu and Whitman, 2008).

Methyl Coenzyme-M Reductase. Methyl coenzyme-M reductase (E.C.2.8.4.1; or coenzyme-B sulfoethylthiotransferase) is one of the key enzymes involved in the methanogenesis pathway (EMBI-EBI, 2016). It is located in the last step of the hydrogenotrophic, methylotrophic, and aceticlastic pathways and is responsible for the conversion of methyl-CoM and CoB to CoM-S-S-CoB and CH_4. Although there are many cofactors and enzymes present in the methanogenesis pathway that are not unique in Archaea, Mcr is believed to be exclusive to methanogenic Archaea, which makes it an enzyme of interest in terms of CH_4 mitigation (Thauer, 1998; Duin et al., 2011; Aguinaga Casañas et al., 2015).

Structure. Methyl coenzyme-M reductase is composed of three different subunits in a $\alpha_2\beta_2\gamma_2$ arrangement (Thauer, 1998; Garbarse, 2005; Shima et al., 2002) with the alpha subunits being encoded by *mcrA*, the beta subunits encoded by *mcrB*, and the gamma subunits encoded by *mcrG* (Hallam et al., 2003). Furthermore, at least one copy of the *mcrA* operon has been found to be present in all methanogenic Archaea genomes, which makes it a gene of interested when identifying these microorganisms (Hallam et al., 2003). Methyl coenzyme-M reductase is also composed of two molecules of a chomophoric prosthetic group, which is a nickel-

porphinoid known as cofactor F_{430}; as well as CoM and CoB when purified (Thauer, 1998).

Mode of action. Methyl coenzyme-M reductase is only active when the Ni in cofactor F_{430} is strongly reduced and in the Ni(I) state (Hedderich and Whitman, 2006; Duin et al., 2016). When in the active state, the enzyme forms a channel using the cofactor F_{430}, which has been demonstrated to extend from the inner protein complex to the exterior protein surface (Elmer et al., 1997; Grabarse et al., 2001; Hedderich and Whitman, 2006; Duin et al., 2016) (Figure 13). Methyl coenzyme-M must first enter the channel, prior to being blocked by CoB, and once this occurs, CoB with the thiol group facing coenzyme F_{430}, will then bind, closing the channel (Hedderich and Whitman, 2006). Within the active site of Mcr, there are five modified amino acids that are responsible for catalysis of the reaction; including methylated histidine, arginine, glutamine, and cysteine side chain residues as well as a glycine residue in which its carbonyl oxygen is substituted with sulfur (Hedderich and Whitman, 2006; Duin et al., 2016). The interior channel of the enzyme is the optimal location for the binding of CoM and CoB due to its hydrophobic environment, unstable free radicals, and the highly conserved nature of the specific amino acid residues (Thauer, 1998; Hedderich and Whitman, 2006).

Alternative hydrogen sinks. Although CH_4 production is one of the major H_2 sinks in the rumen, there are alternative pathways which can utilize and prevent accumulation of H_2. These alternative H_2 sinks include another major H_2 sink, fumerate reduction, and minor H_2 sinks including reductive acetogenesis, nitrate and nitrite reduction, sulfate reduction, and biohydrogenation of unsaturated fatty acids.

Fumarate reduction. The second largest H_2 sink in the rumen is the reduction of fumarate via fumarate-reducing bacteria (Kobayashi, 2010; Ungerfeld et al., 2014). These bacteria, including *Fibrobacter succinogenes*, *Selemonas ruminantium*, *Veillonella parvula* and *Wolinella succinogenes*, can compete directly with methanogens for substrates, including H_2 and formate (Asanuma et al., 1999b; Castillo et al., 2004). Fumarate is first reduced to succinate and then succinate is converted to propionate according to the following overall equations:

$$Fumarate + aqH_2 = Succinate$$

$$Succinate = Propionate + CO_2$$

The formation of propionate within the rumen is a viable alternative use of aqH_2 in which the end-product can be utilized by the animal for energy (Castillo et al., 2004; Ellis et al., 2008).

Reductive acetogenesis. Acetogenic bacteria within the rumen can theoretically compete with rumen methanogens for H_2 as an energy source. These bacteria, such as *Acetitomaculum ruminis*, *Eubacterium limosum*, and *Ruminococcus productus*, reduce CO_2 to acetate using oxidized aqH_2 via the Wood-Ljungdahl pathway (reductive acetogenesis) according to the following overall equation (Fonty et al., 2007):

$$2CO_2 + 4aqH_2 = 3acetate + 2H_2O$$

Although the formation of acetate within the rumen appears to be a viable alternative use of H_2 in which the end-product can be utilized by the animal for energy, this pathway is not commonly employed within the rumen (Ungerfeld, 2015a). The populations of acetogens often dominate the rumen of day old lambs in regards to H_2-utilization and can account for 25% of fermentation output in methanogen-free lambs

(Fonty et al., 2007); though they are quickly over populated by methanogen populations within the first week of life (Morvan et al., 1994; Lopez et al., 1999). Previous research has reported acetogen populations to range from 10^3 to 10^7/ g (depending on the diet); however, they are still 10-fold less than the competitive methanogens in adult animals (Le Van et al., 1998; Fonty et al., 2007; Morgavi et al., 2010). The shift in hydrogenotrophic populations within the rumen is most likely due to differences in partial pressure of H_2 requirements between the acetogens and methanogens (Fonty et al., 2007). Under normal ruminal conditions, when CH_4 is uninhibited, methanogens and acetogens have relatively similar ΔG_T between the reactants and products, being -23.5 and 0.1 kJ/mol, respectively, and deemed to be thermodynamically unfeasible (Kohn and Boston, 2000). However, the partial pressure of H_2 for these reactions to occur are different, with acetogens requiring higher concentrations of H_2 when compared to methanogens (Figure 12; Ellis et al., 2008). When methanogens are present in the rumen, the partial pressure of H_2 is kept consistently low, and below the threshold required for reductive acetogenesis to occur (Ellis et al., 2008; Morgavi et al., 2010). It was therefore postulated that these acetogens rely on alternative energy sources other than CO_2 and H_2 (Joblin, 1999) which is supported by the fact that acetogens are present with methanogens (Henderson et al., 2010). Previous research has also demonstrated that even when acetogen populations are inoculated at ten times the methanogen population in the rumen, they can still not outcompete methanogens or lower CH_4 production (Le Van et al., 1998; Lopez et al., 1999). Only when methanogens are absent and CH_4 production is completely eliminated from the rumen is the partial pressure of H_2 increased above the threshold requirement for acetogens, allowing reductive

acetogenesis to occur, which is demonstrated by an increase in acetate production (Lopez et al., 1999; Fonty et al., 2007; Ungerfeld, 2015b). Theoretically, if CH_4 production is completely inhibited and reductive acetogenesis was to occur, we would expect to see an increase in acetate production. However, a recent meta-analysis of the shifts in the H_2 sinks in methanogenesis-inhibited ruminal fermentation in batch and continuous-cultures showed this was not the case (Ungerfeld, 2015a). Ungerfeld (2015a) proposed that one of the H_2 sinks that was absorbing the "missing H_2" was acetate via reductive acetogenesis; although the study did not report an increase in acetate concentration, suggesting that the H_2 was utilized in other H_2 sinks.

Nitrate and nitrite reduction. The reduction of NO_3^- and NO_2^- is considered a minor H_2 sink within the rumen (Morgavi et al., 2010) due to low NO_3^- concentrations in normal functioning rumens. Unless NO_3^- is supplemented in the ration, very little NO_3^- is present within the rumen unless it is consumed within the forage itself. Nitrate accumulation can occur in forages and is influenced by forage maturity, soil conditions, and fertilizer application (Leng, 2008; Lee and Beauchemin, 2014). This NO_3^- can have a substantial effect on the animal consuming the feed. Nitrate and NO_2^- reducing bacteria can use H_2 as a source of energy and compete with methanogens. These bacteria, including *Wolinella succinogenes*, *Escherichia coli*, *Selenomonas ruminantium*, *Campylobacter fetus*, *Mannheimia succiniciproducens*, and *Veillonella parvula*, can convert NO_3^- to ammonia (**NH_3**) via dissimilatory (energy generating) or assimilatory (energy consuming) nitrate reduction or to N_2O via denitrification (Morgavi et al., 2010; Lin et al., 2011; Latham et al., 2016). Although all three pathways are possible within the rumen, dissimilatory nitrate reduction is the most common due to the fact that the accumulation of NH_3 in the rumen inhibits

assimilatory nitrate reduction (Moreno-Vivian et al., 1999) and bacterial denitrification genes only account for 7% of the genes involved in nitrogen metabolism and therefore this pathway is deemed inconsequential (Brulc et al., 2009; Latham et al., 2016). Therefore, in the rumen, NO_3^- is reduced to NO_2^- and then to NH_3 via dissimilatory reduction according to the following overall equations (Leng, 2008):

$$NO_3^- + 2H^+ = H_2O + NO_2^-$$

$$NO_2^- + 6H^+ = H_2O + NH_3$$

The reduction of NO_3^- to NO_2^- ($\Delta G'°$= -163.2 kJ/mol) and then NO_2 to NH_3 ($\Delta G°$= -436.4 kJ/mol) is more energetically favorable than the reduction of CO_2 to CH_4 ($\Delta G°$= -131 kJ/mol) (Thauer et al., 1977). Ammonia can serve as a nitrogen source and be used for microbial growth; however, the conversion of NO_3^- to NO_2^- is rapid, but the conversion of NO_2^- to NH_3 is slow, which can cause an accumulation of NO_2^- within the rumen (Iwamoto et al., 1999; Morgavi et al., 2010). Nitrite accumulation is common when NO_3^- is supplemented to un-adapted animals; the NO_3^- can then be absorbed across the rumen wall and lead to toxicity and possible death (Leng, 2008). The specific inclusion rates and associated effects of nitrate supplementation will be discussed in detail in the following section regarding CH_4 mitigation strategies.

Sulfate reduction. Similar to the reduction of nitrate and nitrite in the rumen, the reduction of sulfate (**SO_4^{-2}**) is also considered a minor H_2 sink within the rumen (Morgavi et al., 2010) due to low SO_4^{-2} concentrations in normal functioning rumens. Unless SO_4^{-2} is supplemented in the ration, very little SO_4^{-2} is present within the rumen. Naturally occurring sources of SO_4^{-2} include feeds with sulfur-containing amino acids, such as methionine and cysteine (cystine), homocysteine, and taurine;

and B-vitamins including thiamin and biotin that are synthesized by rumen microbes (NRC, 2001; Leng, 2008) and can be found at relatively high concentrations in byproduct feeds (NRC, 2001). This SO_4^{-2} can have a substantial effect on the animal consuming the feed. Sulfate reducing bacteria can use H^+ as a source of energy and actually outcompete with methanogens for substrates when sulfur-containing compounds are not limited in the diet (Zinder, 1993; Ellis et al., 2008). Compared to methanogens and acetogens, sulfate-reducers have more competitive advantages in the rumen, such as a lower minimum H_2 theshold and have a higher affinity for H_2 (lower K_m = 2 µM) (Figure 12; Zinder, 1993; Ellis et al., 2008). Although little information is known about these species and their small populations in the rumen, they still have the ability to have a large impact on ruminal fermentation (Ellis et al., 2008). These bacteria from the *Desulfovibrio* and *Desulfotomaculum* genera, also have the ability to use other oxidized forms of sulfur including thiosulfate, sulfite, and elemental sulfur as electron acceptors and, in addition to H_2, they can use organic acids, alcohols, amino acids, and some aromatic compounds as electron donors (Zinder, 1993; Ellis et al., 2008). These organisms convert predominantly SO_4^{-2} to hydrogen sulfide (**H_2S**) via dissimilatory (energy generating) or assimilatory (energy consuming) sulfate reduction (Leng, 2008). Although both pathways are possible within the rumen, dissimilatory sulfate reduction is the most common (Leng, 2008). Therefore, in the rumen, SO_4^{-2} is reduced to sulfite (**SO_3^{-2}**) and then to H_2S via dissimilatory reduction according to the following overall equations (Leng, 2008):

$$SO_4^{-2} + 2H^+ = 2H_2O + SO_3^{-2}$$

$$SO_3^{-2} + 6H^+ = 2H_2O + H_2S$$

Unlike the endproduct NH_3 in nitrate and nitrite reduction, the end product in sulfate reduction, H_2S, is undesirable and can be toxic to the host animal. The specific inclusion rates and associated effects of sulfate supplementation will be discussed in detail in the following section regarding CH_4 mitigation strategies.

Biohydrogenation of unsaturated fatty acids. Unsaturated fatty acids (**UFA**) are toxic to rumen microbes, and are therefore rapidly biohydrogenated to saturated fatty acids (**SFA**) (Jenkins, 1993). When feed enters the rumen, the fat, mainly in the form of triglycerides (esterified fatty acids), is hydrolyzed by lipolytic microorganisms to yield the free form of the fatty acids (**FA**) (NRC, 2001). These free FA, being predominantly unsaturated, are first isomerized and then hydrogenated by rumen microorganisms (Jenkins, 1993; NRC, 2001). The extent of biohydrogenation depends largely on the degree of unsaturation of the FA, the amount of fat in the diet, the frequency of feeding, and the specific bacterial populations within the rumen (Jenkins, 1993; NRC, 2001). This hydrogenation of UFA can serve as an alternative H_2 sink within the rumen, however; this process has only been estimated to account for 1 to 2% of H_2 absorption (Czerkawski and Clapperton, 1984; Jenkins, 1993).

Methane Mitigation Strategies

From both an environmental, as well as economic standpoints, it is in the best interest of cattle producers to decrease CH_4 emissions, limit their contribution to climate change, and improve overall animal feed efficiency (Beauchemin et al., 2008). There are several options for reducing CH_4 emissions, which can be collectively grouped into three broad categories. These categories include increasing animal production via genetics and management, modifications to feeds, feeding management, and nutrition (including intake, roughage type/ quality, forage to

concentrate ratio, feed processing, total mixed rations/ feeding frequency, and precision feeding/ feed analysis), and supplementation with rumen fermentation modifiers (including plant secondary metabolites/ bioactive compounds, organic acids, microbial/ probiotics/ prebiotics, electron receptors, ionophores, dietary fat, and chemical inhibitors). However, this review will only cover dietary fat and chemical inhibitors.

Dietary fat. The use of dietary fat to lower CH_4 emissions is one of the most extensively researched methods to lower enteric CH_4 from ruminants (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Hristov et al., 2013; Patra, 2013; Caro et al., 2016; Jayasundara et al., 2016). In a collective review involving a range of fat sources and diets in beef cattle, dairy cattle, and lambs, it was determined that for every 1% of DMI increase in fat concentration, there was a 5.6% reduction in CH_4 emissions (g/kg of DMI) (Beauchemin et al., 2008). Dietary fat inclusion in the diet of ruminants lowers CH_4 production by a number of ways including; a reduction of fermentation of organic matter, decreased methanogen and protozoal populations, and serving as an alternative H_2 sink via biohydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995; Bunglavan, 2014). However, the extent of reduction of CH_4 emissions due to increasing levels of fat in the diet can be variable and depends on the concentration of fat, the fatty acid composition, and the overall nutrient composition of the diets (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013). In regards to FA profile of the diet, the inclusion of monounsaturated fatty acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) have a profound effect on CH_4 production, with little to no effect with differences in total concentration of saturated fat (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013;

Bunglavan, 2014). Among the various types of fat used in research to potentially lower CH_4 emissions, the use of PUFA has been demonstrated to have the most extensive CH_4 reduction properties (Patra, 2013; Bunglavan, 2014). Among these fats, the most common are soybean oil, linseed oil, and sunflower oil; however, canola oil has recently increased in popularity due to its economic importance and availability, specifically in western Canada, where over 18 MMT of canola are produced annually (Patra, 2013; Statistics Canada, 2016). Although there are few studies using canola oil as a CH_4 inhibitor (Beauchemin and McGinn, 2006a,b; Brask et al., 2013; Pinares-Patiño et al., 2016), when supplemented at around 3-5% of DM *in vivo* in dairy and beef cattle, canola oil lowered CH_4 emissions (g/d and g/kg of DMI) and had no effect on total VFA concentration, no effect or decrease in acetate, no effect or increase in propionate, and no effect or decrease in acetate:propionate ratio. Canola oil also demonstrated variable results regarding effect on DMI and diet digestibilities. Again, the effect of fat on CH_4 emissions and other parameters tends to be variable when basal diets vary in feedstuffs used and nutrient composition.

Chemical inhibitors. Chemical inhibitors can be broadly divided into two groups, those that indirectly effect methanogenesis (non-specific) and those that directly inhibit methanogenesis (specific).

Non-specific. Chemical inhibitors that indirectly inhibit methanogenesis tend to affect the entire microbial population of the rumen and often include nitrocompounds (nitrate, nitrite, nitroethane, and 2-nitropropanol), phosphate, ethylene, acetylene, and halogenated aliphatic hydrocarbons (chloroform and flouroacetate) (Liu et al., 2011a). These chemicals have been demonstrated to affect both Archaea, as well as bacteria, and inhibit CH_4 production by reducing available

substrates, serving as alternative H_2 pathway, altering the membrane proton gradient, inhibiting coronoid enzymes necessary for methyl-CoM reductase function, and inhibiting acetate metabolism (Liu et al., 2011a).

Specific. Chemical inhibitors that directly inhibit methanogenesis include structure analogs of hydroxymethylglutaryl (**HMG-CoA**) and methyl-CoM, both with a similar mode of action (Liu et al., 2011a). HMG-CoA is a unique cofactor found in methanogenic Archaea. Unlike bacteria with membranes made of glycerol esters of long chain fatty acids, methanogenic Archaea have lipid membranes composed of glycerol esters of long chain isoprenoid alcohols (Miller and Wollin, 2001; Liu et al., 2011a). These isoprenoid alcohols are formed by the precursor known as mevalonate, which is produced by the reduction of HMG-CoA via HMG-CoA reductase (Miller and Wollin, 2001; Liu et al., 2011a). Thus, by inhibiting the binding of HMG-CoA to HMG-CoA reductase, the formation of the lipid membrane is inhibited, which eventually will lead to cell stasis and death (Liu et al., 2011a). Therefore, although structural analogs of HMG-CoA do not directly inhibit the pathway for CH_4 formation, they are still considered direct inhibitors of methanogenesis due to the fact that they suppress the populations of methanogenic Archaea and thus lower overall CH_4 production. Structural analogs of HMG-CoA which have been used to decrease methanogenic Archaea populations in the rumen include mevastatin, fluvastatin, and lovastatin (Wollin and Miller, 1999; Miller and Wollin, 2001; Liu et al., 2011a). Both mevastatin and lovastatin supplemented at low concentrations (≥10 nmol/ mL of culture medium) have been demonstrated to decrease CH_4 production in four strains of *Methanobrevibacter* by 50 to 100% *in vitro* over the duration of the experiment (9 d) (Miller and Wollin, 2001). Additionally, the supplementation of mevastatin and

lovastatin did not negatively affect bacterial populations responsible for fermentation of carbohydrates, including *Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens, Fibrobacter succinogenes,* and *Selenomonas ruminantium* (Miller and Wollin, 2001). In another *in vitro* study, fermented rice straw extract containing 97 mg of lovastatin/ g of crude extract and supplemented at 10 and 20 mg demonstrated a 15% and 28% reduction in CH_4 concentration, when compared to the control, respectively, with a decrease in total methanogens, but no detrimental effects of the bacterial populations including *R. albus*, *F. succinogenes,* and *R. flavefaciens* (Jahomi et al., 2013). However, when lovastatin was supplemented at 80 mg/ kg of DM in sheep fed a mainly hay diet, there was no reduction in CH_4 emissions when compared to the control (Klevenhusen et al., 2011).

The second type of chemical inhibitor that directly affects methanogenesis is structural analogs of methyl-CoM. As previously described, methyl-CoM is a unique cofactor found only in methanogenic Archaea, which is directly involved in the last step of methanogenesis (Figure 15; Lui and Whitman, 2008; Liu et al., 2011a). Structural analogs of this molecule bind to Mcr, the enzyme responsible for the formation of CH_4, which impedes methyl-CoM binding and thus prevents the reduction of methyl-CoM to CH_4 (Liu et al., 2011a). Structural analogs of methyl-CoM include 2-bromoethanesulfonate (**BES**), 2-chloroethanesulfonate (**CES**), 2-mercaptoethanesulfonate (**MES**), lumazine, and most recently invented, a nitrooxy organic molecule known as NOP (Liu et al., 2011a; Duval and Kindermann, 2012).

Although promising results have been demonstrated *in vitro*, the use of most indirect and direct chemical inhibitors have been proven to be less effective *in vivo* due to lack of persistency of inhibition effects, toxicity to the host animal, and

negative side effects on milk production (Liu et al., 2011; Knapp et al., 2014). Nitrooxy organic molecules include 3-nitrooxypropanol, 5-Ni-trooxy-pentanenitrile, 5-nitrooxy-pentane, 3-nitrooxy-propyl propionate, 1,3-bis-nitrooxypropane, 1,4-bis-nitrooxybutane, 1, 5-bis-nitrooxypentane, 3-nitrooxy-propy-1-benzoate, 3-nitrooxy-propyl-hexanoate, 3-nitrooxy-propyl 5-nitrooxy-hexanoate, isosorbid-dinitrate, and N-[2-(nitrooxy) ethyl]-3-pyridinecarboxamide, and bis-(2-nitrooxyethyl) ether (Duval and Kindermann, 2012). Nitrooxy organic molecules are believed to serve as methyl-CoM structural analogs (Duval and Kindermann, 2012). However, recent studies have also tested the theory that these molecules lower CH_4 emissions by forming a NO_2^-/ NO_3^- free radical upon binding to the enzyme (Prakash, 2014; Duin et al., 2016). In recent studies used for a US patent, when the previously listed nitrooxy organic molecules were tested for efficacy, NOP (Figure 16) ($C_3H_7NO_4$; molecular weight of 121.1 g/mol), classified as a nitrooxy alkanoic derivative, was demonstrated to lower CH_4 emissions to the greatest extent, with a reduction of 100% when supplemented at 0.05% of dietary DM (6% hay, 37% corn silage, 13% grass silage, 44% concentrate) *in vitro* (Duval and Kindermann, 2012). The formulation of these molecules was also tested via *in vitro* methods by replacing the nitrooxy group with other organic groups (hydroxyl-, alkoxy-, amino-, alkylamino-, and dialkylamino groups or an alkenyl, or a mono- or polyunsaturated alkynyl carbon chain in any isomeric form); however, it was determined that the drastic reduction in CH_4 was only observed when the nitrooxy group was present (Duval and Kindermann, 2012). Follow-up studies regarding the use of NOP as a CH_4 inhibitor have further been conducted *in vitro* (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2015a; Romero-Pérez et al., 2016), as well as *in vivo* in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b;

Vyas et al., 2016a,b), dairy cattle (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015; Haisan et al., 2016; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014), with all studies exhibiting positive results for the use of NOP as a CH_4 inhibitor.

When NOP was supplemented at 8 and 16 mg of NOP/ g of DM *in vitro* using an alfalfa hay and oat (60:40) diet and sheep rumen fluid in a batch culture, there was no effect on total gas production or total VFA concentration; however, there was 86 and 95% reduction in CH_4 and a decrease in the acetate: propionate ratio (Martínez-Fernández et al., 2014). Similar results were observed by Romero-Pérez et al. (2015a) *in vitro* for a diet of 60% barley silage, 35% barley grain, and 5% supplement (DM basis) using beef cattle rumen fluid; there was no effect of including NOP (supplemented at 0.5, 1.0, and 2.0 mg of NOP/ g of DM) on total gas production, but there was a 76, 85, and 86% reduction in CH_4 emissions, respectively. This study also observed an increase in gH_2 production of 159, 205, and 218% with increasing inclusion rates of NOP. Although total VFA concentration was not affected, there was a reduction in the molar proportion of acetate with the highest NOP inclusion (2 mg of NOP/ g of DM) with no effect on propionate proportion or acetate: propionate ratio. It was also reported that there was no effect on total ruminal protozoa, liquid or solid associated bacteria or liquid associated methanogens, but there was a reduction in solid phase methanogens at all concentrations of NOP. In a follow-up study by the same group, the use of NOP (2 mg of NOP/ g of DM) and monensin (MON; 2 mg of MON/ g of DM), and the combination of NOP+MON (2 mg of NOP and 2 mg of MON/ g of DM) was studied (Romero-Pérez et al., 2016). Total gas production was lowered with the supplementation of NOP (13% reduction) and NOP+MON (10%

reduction); however, gH_2 production was increased by 66 and 83% with NOP and NOP+MON, respectively. Methane emissions were lowered by 72, 12, and 70% and there were no effects on the liquid or solid associated bacteria or liquid associated methanogens, but there was a reduction in the solid phase methanogens with NOP, MON, and NOP+MON inclusion, respectively. Overall, regarding the previously published *in vitro* data on the inclusion of NOP as a CH_4 inhibitor; it is clear that this molecule has the potential to lower CH_4 emissions and increase gH_2 production without negatively effecting liquid or solid associated bacterial populations or protozoa, or dry or organic matter digestibilities, with variable results for acetate and propionate concentrations and ratios (which could most likely be explained by logistical differences between batch and continuous culture fermenters).

The inclusion of NOP demonstrated a reduction in CH_4 emissions production and/ or CH_4 yield in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b), dairy cattle (Haisan et al., 2014; ; Reynolds et al., 2014; Hristov et al., 2015; Haisan et al., 2016; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014) (Table 4,5). When NOP was administered to sheep at 100 mg/ d (approximately 110 mg/ kg of DM) and dosed into the rumen immediately prior to feeding, there was no effect on CH_4 production (g/d); however, there was a 24% reduction in CH_4 yield when expressed as g/kg of DMI (Martínez-Fernández et al., 2014). Additionally, when NOP was supplemented at 2,500 mg/ d (approximately 125-130 mg/ kg of DM), Reynolds et al. (2014) only saw a 7% reduction in CH_4 production (g/ kg of DMI) when NOP was supplemented directly into the rumen. On the contrary, when Haisan et al. (2014, 2016) mixed NOP at the same inclusion rate into the total mixed ration (**TMR**) prior to feeding, they saw a 60 and 37% reduction, respectively, in CH_4

production (g/ kg of DMI) in lactating dairy cows. Similarly, Romero-Pérez et al. (2014) only saw a 33% reduction in CH_4 production (g/ kg of DMI) when NOP was supplemented at 2.8 g/d (approximately 346 mg/ kg of DM) and administered as a top dressing to the TMR whereas, yet in a proceeding study by the same group, Romero-Pérez et al. (2015b) supplemented NOP at 2 g/d (approximately 280 mg/ kg of DM) mixed in the TMR and observed a 60% reduction in CH_4 production (g/ kg of DMI). However, it was postulated that the differences in magnitude of CH_4 reduction could have been due to dosing the rumen immediately prior to feeding (Martínez-Fernández et al., 2014; Reynolds et al, 2014) verses top dressing the TMR (Romero-Pérez et al., 2014), verses mixing the product into the TMR (Haisan et al., 2014; Romero-Pérez et al., 2015b), which would affect the amount of time the product was in the rumen (Haisan et al., 2014). Lopes et al. (2016) and Hristov et al. (2015) both reported 31-34% reduction in CH_4 production (g/ kg of DMI) using the GreenFeed system (C-Lock, Inc., Rapid City, SD, USA) when NOP was supplemented at 60 mg/ kg of DM (1.5 and 1.7 mg/d, respectively) and mixed into the TMR fed to medium and high producing lactating dairy cows. Thus, differences in CH_4 reduction may be due to differences in supplementation rate of NOP as well as administration technique.

NOP supplementation has been demonstrated to increase gH_2 production. Lopes et al. (2016) and Hristov et al. (2015) both found gH_2 production to be 0 g/d for the control and 1.27 to 1.30 g/d when NOP was supplemented at 60 mg/ kg of DM and mixed into the TMR in medium and high producing lactating dairy cows. Furthermore, when NOP was supplemented at approximately 280 mg/kg of DM, gH_2 production was found to be 35.9 g/d (Romero-Pérez et al., 2015b). Although the supplementation rate of NOP in the later study was almost five times the amount used

in the previous studies, which would explain some, but not all of the difference, this value was also calculated based on CH_4 and VFA production and was not directly measured as it was in the first two studies.

In regards to ruminal fermentation, it has been reported that the inclusion of NOP did not affect mean ruminal pH or total VFA concentration in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b), dairy cattle (Haisan et al., 2014; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014). However, in one study, with inclusion of NOP at 500 and 2,500 mg of NOP/ d (approximately 25 and 125 mg/ kg of DM), the lower dose lowered and the higher dose increased average ruminal pH and the higher dose also decreased total VFA concentrations (Reynolds et al., 2014). Studies report a decrease in acetate (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Haisan et al., 2014; Lopes et al., 2016; Reynolds et al., 2014) and an increase in propionate concentrations (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Lopes et al., 2016), which led to a decrease in acetate: propionate ratio when compared to the control (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Haisan et al., 2014; Lopes et al., 2016; Reynolds et al., 2014). The inclusion of NOP has also been demonstrated to have no effect on total bacteria populations (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Haisan et al., 2014; Lopes et al., 2016), increased (Romero-Pérez et al., 2015b) or had no effect on protozoa populations (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Haisan et al., 2014), and decreased (Romero-Pérez et al., 2015b; Haisan et al., 2014) or no effect on methanogens

populations (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Lopes et al., 2016).

When NOP was supplemented *in vivo* to sheep (Martínez-Fernández et al., 2014), dairy cattle (Haisan et al., 2014; Hristov et al., 2015; Lopes et al., 2016; Reynolds et al., 2014), and beef cattle (Romero-Pérez et al., 2015b), there was no effect on DMI between the control and NOP-treated animals. Except for one study (Romero-Pérez et al., 2014), DMI only differed from the control when NOP was supplemented at 2.25 mg/ kg of body weight (**BW**) (approximately 1.4 g/ d or 163 mg/ kg of DM), with both lower and higher doses of NOP being similar to the control DMI values. The inclusion of NOP in the diets also had similar digestibilities of DM, OM, CP, NDF, ADF, and starch (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2014; Hristov et al., 2015), except for in one study when NOP was supplemented at 500 and 2,500 mg of NOP/ d (approximately 25 and 125 mg/ kg of DM) (Reynolds et al., 2014). In that study, supplementation of NOP at the higher level caused lowered DM, OM, ADF, and CP digestibilities and supplementation of NOP at the lower level caused lowered NDF and ADF digestibilities. However, these four previously mentioned studies administered the NOP treatment differently. Martínez-Fernández et al. (2014) and Reynolds et al. (2014) both administered the NOP treatments directly into the rumen via the cannula immediately before feeding; Romero-Pérez et al. (2014) top dressed the NOP treatment onto the TMR; and Hristov et al. (2015) mixed the NOP treatment directly into the TMR, which could explain variations in diet digestibilities.

In lactating dairy cow studies, the inclusion of NOP in the diets had no effect on milk yield, fat corrected milk (FCM)/energy corrected milk (ECM) yield, or feed

efficiency (FE) (Haisan et al., 2014; Hristov et al., 2015; Lopes et al., 2016; Reynolds et al., 2014). The milk components data were more variable, with some studies reporting no effect on components yield (Hristov et al., 2015; Reynolds et al., 2014) and no effect on components proportion (Haisan et al., 2014; Hristov et al., 2015). Reynolds et al. (2014) reported an increase in protein proportion and other studies reported an increase in milk fat proportion (Lopes et al., 2016), and an increase in short chain fatty acids (Hristov et al., 2015) or no effect on fatty acid profile in milk (Reynolds et al., 2014) with NOP supplementation.

Conclusion

The natural capabilities of the Earth's GHE has been intensified due to an accumulation of small molecules in the atmosphere that has caused an increase in the Earth's surface temperature and if it increases significantly, it will make the planet uninhabitable (Sejian and Naqvi, 2012). There are numerous more complex molecules that comprise the atmosphere and increase the Earth's GHE and can affect the balance of energy exchange between the atmosphere, space, land, and oceans (EPA, 2015). These molecules are collectively known as GHG and include; H_2O vapor, CO_2, CH_4, N_2O, and other trace gases. Methane is the second most prevalent GHG emitted by anthropogenic sources, with anthropogenic emissions accounting for approximately 70-80% of the total CH4 emissions (EPA, 2015; Johnson and Johnson, 1995). The Agriculture sector is the second largest source of anthropogenic GHG emissions in the United States, contributing 515.70 MMT CO2 Eq. and representing 7.73% of the total GHG emissions (IPCC, 2013; EPA, 2015). Methane production from enteric fermentation made up 25.85% of CH_4 emissions and was the single largest contributor to total CH_4 production in the United States in 2013 (IPCC, 2013; EPA, 2015). Not

only do CH_4 emissions negatively influence the GHE and GW of the Earth, CH4 emissions from ruminants can account for two to 12% GEI loss for the animal, depending on animal management (beef or dairy), stage of production (calves, heifer replacements, stockers, lactating or dry cows, or bulls), level of feed intake, and composition of the diet (forage or concentrate) (Johnson and Johnson, 1995; Beauchemin et al., 2009; EPA, 2015).

From both an environmental, as well as economic standpoints, it is in the best interest of cattle producers to decrease CH_4 emissions, limit their contribution to climate change, and improve overall animal feed efficiency (Beauchemin et al., 2008). A novel feed additive, NOP, as a CH_4 inhibitor has been studied in vitro (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2015a; Romero-Pérez et al., 2016), as well as in vivo in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Vyas et al., 2016a,b), dairy cattle (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015; Haisan et al., 2016; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014), with all experiments exhibiting positive results for the use of NOP as a CH_4 inhibitor. Additionally, the use of dietary fat to lower CH_4 emissions is one of the most extensively researched methods to lower enteric CH_4 from ruminants (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Hristov et al., 2013; Patra, 2013; Caro et al., 2016; Jayasundara et al., 2016). In a collective review involving a range of fat sources and diets in beef cattle, dairy cattle, and lambs, it was determined that for every 1% of DMI increase in fat concentration, there was a 5.6% reduction in CH_4 emissions (g/kg of DMI) (Beauchemin et al., 2008). Therefore, more research is warranted to determine the effect of NOP and unsaturated fatty acids alone and in combination on their ability to reduce methane emissions.

Chapter 2

THE EFFECT OF 3-NITROOXYPROPANOL AND CANOLA OIL ALONE AND IN COMBINATION ON METHANOGENEISIS IN BEEF CATTLE

Objective

The objective of this study was to determine if a novel feed additive (3-nitrooxypropanol) and unsaturated fat (canola oil) alone and in combination could mitigate CH_4 gas emissions from cattle. Additionally, the effects of this feed additive and unsaturated fat on related parameters including diet digestibility, rumen fermentation, microbiome characterization, and abundance of methyl coenzyme M reductase (mcrA) genes were observed.

Hypothesis

I hypothesized that supplementation of a diet with a novel feed additive (3-nitrooxypropanol) and unsaturated fat (canola oil) individually would lower CH_4 emissions and the combination of these two supplements would further decrease CH_4 emissions compared to individual supplementation of these additives.

Materials and Methods

Animals and Diets**.** This experiment was a joint collaboration between Dr. Limin Kung, Jr. (University of Delaware, Newark, Delaware, USA) and Dr. Karen A. Beauchemin (Agriculture and Agri-Food Canada Lethbridge Research and Development Centre, Lethbridge, Alberta, Canada). This study (KB1607) was conducted at the Metabolism Unit and Controlled Environment Building at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (Lethbridge, Alberta, Canada) and all procedures were approved by the Lethbridge

Research and Development Centre Animal Care Committee (LRDC-ACC) (ACC Protocol #1607) and conducted in accordance with the guidelines set forth by the Canadian Council on Animal Care (CCAC) (1993). Eight previously ruminally canulated beef heifers (Angus cross, 732 ± 43 kg) were used in a double 4 × 4 Latin square design with four 28-d periods and four dietary treatments. The dietary treatments were: 1) control (**CON**) (no supplementation of 3-nitrooxypropanol or canola oil), 2) canola oil alone (**OIL**) (5.0% of diet DM), 3) 3-nitrooxypropanol alone (**NOP**) (200 mg/kg of diet DM; CLOU-3, DSM Nutritional Products Ltd., Kaiseraugst, Switzerland), and 4) 3-nitrooxypropanol (200 mg/kg of diet DM) and canola oil (5.0% of diet DM) combined (**NOP+OIL**).

Due to facility and equipment restrictions, animals were divided into two groups and their start dates were staggered by 1 week to accommodate the number of animals in the experiment. Animals were blocked according to body weight into two groups (Group 1- 738 ± 33 kg and Group 2- 726 ± 50 kg) and then into four pairs (Pair 1- 720 ± 19 kg, Pair 2- 749 ± 6 kg, Pair 3- 715 ± 74 kg, and Pair 4- 744 ± 26 kg) and randomly assigned to one of the four treatments according to statistical blocking guidelines for experiments designed with residual effects when treatments were applied in sequence (Cochan and Cox, 1957) to balance for carry-over effects (Table 6).

All animals were removed from the feedlot and transported to a barn where they were bathed, treated for lice, assigned to an individual tie stall, and allowed to acclimate to the facility for a minimum of 2 weeks before the start of the experimental periods (designated as the Training Period) (Table 7). During this time, all animals received a basal diet comprised of 90.00% barley silage, 4.12% dry rolled barley

grain, 0.88% treatment (placebo) mix and 5.00% supplement (Table 8) which was based on requirements for maintenance set forth for beef cattle with 732 kg of body weight (NRC, 1996) and formulated using a prototype beef cattle feed ration formulation software (National Research Council Nutrient Requirements of Beef Cattle Version 1.0.10 (March 2014), Washington, DC USA) (Table 9). Net energy for maintenance (NE_m) and net energy for maintenance intake (NE_m intake) were calculated based on the average animal body weight of both groups of animals (so that all animals received the same amount of feed on a DM basis) using equations defined by the NRC (1996). For both groups, the NE_m intake was calculated to be 7.60 kg of DM/ d.

Animals in this experiment were fed a high forage diet (90% forage and 10% concentrate) with a restricted level of NE_m intake to both maximize CH_4 emissions and limit growth. The high inclusion rate of forage in the ration was targeted to create a ruminal environment that would favor H_2-yielding fermentation pathways (acetate) as opposed to H_2-consuming pathways (propionate and butyrate), which would therefore produce the most CH_4, and provide a challenge for the treatments to lower emissions. Due to the size of the animals at the start of the Training Period (732 kg), it was imperative that these animals did not increase in physical mass which could cause complications while in the enteric fermentation chambers, metabolism stalls, cattle chutes, and while walking onto the scale. Animals were therefore limit-fed to supply maintenance energy requirements, as opposed to providing *ad libitum* intake.

The barley silage used in this experiment was a 50:50 w/w ratio of two varieties mixed together in order to limit lodging. The two varieties were “CDC Coalition” (TR03373, Canterra Seeds, Winnepeg, MB Canada) and “Chigwell”

(BT577, SeCan Association, Kanata, ON Canada) and both were distributed by Haney Farms (Picture Butte, AB Canada). All other grains in this experiment were locally sourced from the Southern Alberta region. Ingredients were mixed daily in a Calan Super Data Ranger (American Calan, Inc., Northwood, NH USA) equipped with a scale (model #1015, Avery Weight-Tronix, Fairmont, MN USA) and fed at approximately 10:00 am. All animals received a basal diet with one of two treatment mixes, containing either 8.85% 3-nitrooxypropanol on 60% silicon dioxide and 40% propylene glycol (to supply 3-nitrooxypropanol at 200 mg/kg of diet DM) (NOP and NOP+OIL treatments) or a placebo containing 60% silicon dioxide and 40% propylene glycol (CON and OIL treatments). Both treatment mixes were mixed weekly and refrigerated prior to use to maintain freshness. Due to the specific order of the addition of feed ingredients to the Data Ranger, a standard operating procedure was developed and used by all farm crew staff responsible for feeding. First, barley silage was added into the Data Ranger. Dry-rolled barley grain, supplement mix, and both treatment mixes were pre-weighed individually on a small scale (model PB3002-S, Mettler-Toledo, Inc., Lethbridge, AB Canada) and added to the top of the silage mass in the Data Ranger in the previously listed order. The TMR was mixed for a total of two minutes on high speed. Canola oil was supplemented at 5.00% of diet DM (OIL and NOP+OIL treatments). The canola oil was pre-weighed prior to feeding and was added to the total mixed ration (**TMR**) by pouring it directly to the Data Ranger. The TMR was mixed for another two minutes before being weighed out for each animal.

To ensure adequate and even distribution of canola oil over the entire TMR, prior to the start of the experimental periods, a mock TMR was made in the Data

Ranger for 2 animals (approximately 40 kg of feed, as fed) prior to the Training Period. The canola oil (760 g total) supplemented to the TMR was dyed red (High Visibility Foam Colorant, Loveland Industries, Inc., Loveland, CO USA) for easier visibility of the oil. The oil was added to the TMR in the Data Ranger while mixing and was mixed at high speed for a total of two minutes once all of the oil had been poured into the machine. When the TMR containing the dyed canola oil was expelled from the Data Ranger, the oil was equally distributed thoughout the entire TMR mass and was deemed as an adequate method for applying and mixing the oil into the TMR.

Feed offered was measured via a portable flat scale (model FV-150, Mettler-Toledo, Inc., Lethbridge, AB Canada) and feed refused was measured using a Ranger Mate Feed Cart (American Calan, Inc., Northwood, NH) equipped with a scale (model #1015, Avery Weight-Tronix, Fairmont, MN USA). Feed offered and refused was recorded every day to calculate as-fed intakes. Animals were exercised daily in a 38 × 12 m dirt paddock from approximately 0700 to 1000 (except during the enteric fermentation measurements and during the total collection for digestibility of nutrients).

During d 1-14 of each period, all animals were adapted to their respective treatment prior to the initiation of sampling.

Sampling and Chemical Analysis**.**

Feed. Feed samples (TMRs, barley silage, dry-rolled barley grain, supplement, and treatment (placebo or NOP) mixes were obtained during Week 1 (d 7), Week 2 (d 14), Week 3 (d 18 to 20), and Week 4 (d 24 to 27) of every Period. Canola oil (250 mL) was sampled during Week 1 (d 7) of every Period and frozen at -80 °C. Samples of TMRs and barley silage were obtained for DM analysis and used to calculate DMI

during Weeks 1 and 2. Samples of TMRs and barley silage were composited into a single sample for Week 3 and a single sample for Week 4 for every Period and used to calculate DM, DMI during the enteric fermentation measurements and during the total collection for digestibility of nutrients, and for chemical analysis. Dry-rolled barley grain, supplement and treatment mixes were composited into a single sample for each Group during each Period and used to calculate DM and for chemical analysis. Four liters of composited barley silage or composited TMRs, 4 L of composited dry-rolled barley, and 2 L of composited supplement mix, and 1 L of treatment mix were processed using either a 10 cm (for TMRs and forages) or 2.5 cm (for grains, supplement, and treatment mixes) homemade feed splitter, yielding two identical piles. One pile was retained as a composite back-up sample and frozen at -20 °C. The other pile was split again by using the respective splitter, one pile was either retained for particle size distribution analysis (TMRs and barley silage), retained for processing index score and particle size distribution (dry-rolled barley grain), retained for NOP analysis (treatment mixes), or discarded (supplement mix). The other pile was weighed into a pre-weighed paper bag and dried at 50 °C for 96 h in a horizontal air flow oven (VWR model #1690, Sheldon Manufacturing, Inc., Cornelius, OR USA) for determination of DM content. After dried samples were removed from the oven, they were allowed to cool to room temperature before the final weights were obtained and recorded. All dried samples were ground to 4 mm in size (SM 100, Retsch Inc., Haan, Germany). The 4 mm ground sample was then split in two and one sample was retained as a back-up and the other was ground to 1 mm in size and used for chemical analysis.

Dry matter intake. Dry matter intakes (DMI) were calculated daily for each animal using daily as-fed intakes and orts and the DM value obtained on the last day of the Training Period (d 0) and Week 1 (d 7) and Week 2 (d 14) and the DM obtained from the composited TMR samples of Week 3 (d 18 to 21) and Week 4 (d 24 to 27) of every Period, respectively.

Chemical analysis. Feed ingredients and TMRs were ground to 1 mm in size and analyzed for analytical dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude fat (ether extract (EE)), gross energy (GE) and a subsample was ground to ~5 µm in size at a frequency of 30 Hz/ s for a duration of 2 min 30 s grinding time (MM 400, Retsch Inc., Haan, Germany) and analyzed for crude protein (CP) and starch. All samples were weighed using balances connected to computers equipped with serial data acquisition software (CPS Plus Terminal Professional Release V. 5.5.1- Production, ProgramBL.com).

Analytical DM and OM analysis was conducted according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 350.2001.00, Title: Analytical Dry Matter and Organic Matter: Hot Weighing Method, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) using a hot weighing technique which has been accepted as a more rapid weighing procedure and is subject to less error when compared to using desiccators (Van Soest and Robertson, 1985). A balance (model AE200, Mettler-Toledo, Inc., Lethbridge, AB Canada) was heated with a warm crucible (CoorsTek® High Form Ceramic Crucible, 30 mL, Sigma-Aldrich Canada Co., Oakville, ON Canada) prior to weighing and checked for accuracy with a crucible of known weight and the balance was set for a long measuring cycle (for

unfavorable ambient conditions) and the least sensitive setting for stability. Prior to weighing of sample, clean, dry crucibles were warmed in an oven (model 655F, Fisher Isotemp Oven, Thermo Fisher Scientific Co., Toronto, ON Canada) at 105 ± 5°C for a minimum of 30 min. Crucibles were removed individually from oven, weighed, and the stable (lowest) weight was recorded. 1 ± 0.05 g of sample was weighed into each crucible and immediately placed in pre-heated oven. Samples were dried at 135 ± 5°C for 2 h (AOAC Official Method 930.15) (AOAC International, 1995) and then allowed to rest for 15 min at 105 ± 5°C prior to final weighing to ensure crucibles were at equilibrium with sample and analytical DM was calculated. Crucibles containing samples were then put into a pre-heated muffle furnace (model 30400 Thermolyne Furnace, Barnstead International, Dubuque, IA USA) for determination of OM. Samples were burned in reference to a modified AOAC Official Method 942.05 in which the samples were ashed at 550 ± 20°C for a duration of 5 h instead of 600°C for a duration of 2 h (AOAC International, 1995) and then weighed using previously described techniques and OM was calculated. Any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run.

Feed samples were analyzed for NDF and ADF according to Van Soest et al. (1991) and AOAC International (1995) (AOAC Official Method 973.18), respectively, and according manufacturer's instructions (Ankom Technology, 2014a; Ankom Technology 2014b) using 25 µm bags (ANKOM F57 Fiber Filter Bags) labeled with a marking pen (model F08 solvent and acid resistant pen) and closed with a heat sealer (model 1915) and fiber analyzer (ANKOM^{200} Fiber Analyzer, Ankom Technology, Macedon, NY USA). A 500 ± 0.05 mg sample was used and due to the high concentrations of fat (> 5% of diet DM) in diets containing OIL (OIL and NOP+OIL)

and treatment mixes (placebo and NOP), all samples were subject to an acetone pre-rinse for 10 min prior to extraction. A blank standard and a known NDF/ ADF standard sample (FL01.08 Alfalfa hay) were analyzed every 10 samples and used for quality control. Neutral detergent fiber and ADF were then extracted and the concentrations were calculated. Any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run.

Nitrogen analysis was conducted according to the Dumas method of AOAC International (1995) (AOAC Official Method 968.06). Ground feed samples (~5µm in size) were analyzed in duplicate for nitrogen content by weighing 5.0 ± 0.05 mg of sample into 8 × 5 mm pressed tin capsules (D1008, Elemental Microanalysis Ltd., Okehampton, UK). Standard nitrogen samples with 1 ± 0.05, 3 ± 0.05, 5 ± 0.05, and 8 ± 0.05 mg of soft spring wheat standard (B.M.O.) were analyzed every twenty-four samples for determination of a concentration curve. A reference nitrogen sample with 5 ± 0.05 mg of soft spring wheat standard (B.M.O.) of known nitrogen concentration was analyzed every twelve samples for determination of quality control and any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run. Samples were analyzed using an elemental analyzer (NA 2100 Elemental Analyzer, Carlo Erba Instruments, Milan, Italy) which combusted the samples at 1020 °C and the gases were then reduced to N_2 in the presence of Cu at 750 °C. Nitrogen was separated using a stainless steel porous polymer chromatography column (3 m × 6 mm × 5, Poropak QS, mm Agilent Technologies Canada Inc., Mississauga, ON Canada) using CO_2 as the carrier gas and detected using a terminal conductivity detector. Crude protein was calculated from nitrogen concentration values.

Starch analysis was conducted according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 3446.6001.00 Title: Starch Analysis, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) according to methods by Bach Knudsen (1997) and a modified method of Herrera-Saldana et al. (1990) in which 200 µL of amyloglucosidase (*Aspergillus niger*) (Megazyme Inc., Chicago, IL USA) was used instead of 100 µL of glucoamylase and the second incubation had a duration of only 120 min instead of 14 h. Ground feed samples (~5µm in size) were analyzed in duplicate for starch concentration using 200 ± 0.05 mg for TMRs and forages, 150 ± 0.05 mg for the treatment mixes and supplement mix, and 120 ± 0.05 mg for the dry-rolled barley samples. A blank, standard starch solutions with 10 ± 0.05, 20 ± 0.05, 40 ± 0.05, 60 ± 0.05, 80 ± 0.05, and 100 ± 0.05 mg of dextrose, and a Quality Control (QC) starch solution with 80 ± 0.05 mg of corn starch were analyzed every twenty samples for determination of quality control and any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run. Optical density of blanks, standards, and samples were measured at 508 nm using a microplate reader (Dynatech MRX Microplate Reader, Dynatech Laboratories Inc., Alexandria, VA USA) according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 3130.000 Title: Microplate Reader: Operation of Dynatech MRX, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and starch concentration was calculated.

Total mixed rations from Week 4, barley silage, dry-rolled barley grain, supplement, and treatment mixes were composited by weight by Period and analyzed for crude fat. Crude fat concentration was analyzed according to guidelines set forth

by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 350.2010.00 Title: Crude Fat: Ether Extraction Method, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) in reference to previously accepted methods (AOAC Official Method 920.39) (AOAC International, 1995). Samples were analyzed in duplicate using a sample size of 2.0 ± 0.10 g. Extraction was conducted using a 6-flask extraction unit equipped with glass thimble holders and ether reclaiming tubes (model E-816 Hot Extraction (HE), BÜCHI Labortechnuk AG, Flawii, Switzerland) with an extraction time of 300 min at 100% heat (190 °C), a rinse of 30 min at 100% heat (190 °C), and a drying period of 5 min at 100% heat (190 °C). A Quality Control (QC) crude fat standard with 2.0 ± 0.10 g of corn distillers grain was analyzed in duplicate in every run for determination of quality control and any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run.

Composited TMRs from Week 3 and Week 4 and composited canola oil were analyzed for fatty acid profile. For fatty acid analysis of the TMRs, samples were covered with two layers of cheesecloth, frozen at -20°C, and then freeze dried at -30°C for a duration of 14 d (VirTis Sentry Series 981, SP Industries, Inc., Gardiner, NY USA). Samples were ground to ~5 µm in size at a frequency of 30 Hz/ s for a duration of 2 min 30 s grinding time (MM 400, Retsch Inc., Haan, Germany).

Fatty acid profile was analyzed at the Meat Lipid Research Laboratory of the Lacombe Research and Development Centre (Agriculture and Agri-Food Canada, Lacombe, AB Canada). Fatty acids were directly derivatized as fatty acid methyl esters (FAME) and analysis was conducted according to guidelines set forth by the Meat Lipid Research Laboratory (Food Safety and Quality) Section of the Lacombe

Research and Development Centre (SOP Code: PREP-FAME-INSITU-FEED-RNMLAB Title: In situ Methylation and Transmethylation of Feed According to RNM Laboratory Method, Agriculture and Agri-Food Canada, Lacombe, AB Canada) according to modified methods by Sukhija et al. (1988) in which GLC15A (6% C16:0, 3% C18:0, 35% C18:1 (methyl oleate), 50% C18:2, 3% C18:3, and 3:C20:0) and GLC603 (100% C18:1 (methyl vaccenate) (NU-CHEK-PREP, Inc., Elysian, MN USA) were used as standards instead of Mixture Me 61 (4% C4:0, 2% C6:0, 1% C8:0, 3% C10:0, 4% C12:0, 10% C14:0, 2% C14:1, 25% C16:0, 5% C16:1, 10% C18:0, 25% C18:1, 3% C18:2, 4% C18:3, and 2% C20:0) (Larodan Fine Chemicals AB, Malmö, Sweden); diluted 3 N methanolic hydrochloric acid was used as a derivatizing agent instead of acetyl chloride added to anhydrous methanol; 100 µL of internal standard (C17:1 *cis* 10 methyl ester, NU-CHEK-PREP, Inc., Elysian, MN USA) in hexane (4 mg / mL) was used instead of 2 mL of 0.2 mg C21:0 or C23:0 or C19:1/ mL toluene as an internal standard; centrifugation at 1000 × *g* for 30 min instead of at 1500 rpm for five min was used to separate layers of solution; and FAME extract was purified using this layer chromatography (TLC) instead of 1 g of sodium sulfate and 1 g of charcoal. Specifically, 100 ul of internal standard was added to either 50 mg freeze dried ground feed or 20 mg of canola oil. Samples were dual methylated (i.e. base followed by acid) using reagents described by Kramer et al. (1997) to avoid isomerization of conjugated linoleic acid isomers. Two mL 0.5 N sodium methoxide (Supelco Sigma-Aldrich, St Louis, MO USA) in methanol was added and samples were heated for 10 min at 50 ^{o}C. Samples were cooled to room temperature followed by addition of 2 mL 3 N methanolic HCl (Supelco, Bellefonte, PA USA) in methanol, with further methylation at 50 ^{o}C for 10 min. Samples were cooled to room

temperature, and 1.5 mL hexane and 5 mL water added, shaken vigorously for 30 seconds, and centrifuged (5 min at 1000 × *g*). The upper (hexane) layer containing fatty acid methyl esters (FAME) was transferred to 2 mL vials. Completeness of methylation was monitored using thin layer chromatography on silica gel G plates. The TLC plate was developed in a solution with a ratio of 85:15:1 of hexane: ethyl ether: acetic acid and sprayed with 0.01% 2',7'-dichlorofluorescein in methanol (w/v) solution.

Gas chromatography (GC) was conducted using using a Varian CP3800 GC equipped with a 1079 injector, 8400 autosampler, flame ionization detector (Varian Inc, Palo Alto, CA USA) and an SP-2560 capillary column (100 m, 0.25 i.d., 0.2 µm film thickness; Supleco Sigma-Aldrich, St Louis, MO USA). Samples (1 µL) were injected using a 20:1 split, injector and detector temperatures of 250 ^{o}C, with $H_{2(g)}$ as the carrier gas at a constant pressure of 25 psi (initial flow rate of ~1 mL/min). The 175 ^{o}C plateau temperature program described by Kramer et al. (2008) was used for GC analysis. Identifications of FAME were made using reference standards GLC15A and GLC603 Branched-chain FAME were identified using reference standard BC-Mix1 (Applied Science Laboratories, State College, PA USA). For conjugated linoleic acid (CLA) isomers, the UC-59M standard (NU-CHEK-PREP, Inc., Elysian, MN USA) was used which contained all four positional CLA isomers. *Trans*-C18:1, CLA isomers and other biohydrogenatoin intermediates not included in the standard mixtures were identified by their retention times and elution orders as reported in literature (Cruz-Hernandez et al. 2004, Kramer et al. 2008 and Gómez-Cortés et al. 2009) and this included recently identified Δ-9 desaturation products of *t*18:1 isomers (Vahmani et al. *in press*).

Gross energy. The energy content of the TMRs during Week 4 were determined according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 3446.6001.00, Title: E2K Calorimeter Procedure for Determination of Energy Value, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and manufactures' methods of analysis (Digital Data Systems (Pty) Ltd., Gauteng, South Africa). Subsamples of TMRs with 0.300 – 0.350 g of sample was transferred into a gel capsules (CAL2K-4-GC, Digital Data Systems (Pty) Ltd., Gauteng, South Africa). Two blanks and two QC standards (benzoic acid) were also analyzed. All samples, blanks, and standards were analyzed for energy content using an isothermal bomb calorimeter (E2K) equipped with a combustion calorimeter, a cooler (CAL2K-2), and a filling station with regulators (CAL2K-3, Digital Data Systems (Pty) Ltd., Gauteng, South Africa). Canister was pressurized to 1,500 psi with oxygen and the firing voltage was set at 25 volts. Gross energy was calculated.

Particle size distribution. Particle size distribution was determined on forages, TMRs, and dry-rolled barley grain. Particle size was analyzed on a composited barley silage sample from Week 1 (d 7), Week 2 (d 14), Week 3 (d 18) and Week 4 (d 24) and on TMRs during Week 3 (d 18 to 20) and Week 4 (d 24 to 27) of every Period using the Penn State Particle Separator according to Kononoff et al. (2003). Particle size was analyzed on composited dry-rolled barley grain and whole barley grain samples from Week 1 (d 7), Week 2 (d 14), Week 3 (d 18) and Week 4 (d 24) of every Period using a vertical shaking apparatus (Rotap Shaker model RX-29, W.S. Tyler, Mentor, OH USA) equipped with U.S.A. standard brass testing sieves (8 inches in diameter) with mesh openings including 4.00 mm, 2.36 mm, 1.18 mm, and 850 µm, and a bottom pan

(Thermo Fisher Scientific Co., Toronto, ON Canada). Each sample was shaken for a total of five minutes. All samples were analyzed for particle size distribution in duplicate and each forage, TMR, or grain was averaged together for a single value for the Period.

Processing index. Processing index (PI) (measure of bushel weight (lbs./bushel) after processing as a percentage of bushel weight (lbs./bushel) before processing) (Rode and Beauchemin, 1998) was obtained for composited dry-rolled barley grain and whole barley grain samples at the end of each Period using bushel weigh apparatus including a 0.5-liter cup, Cox funnel, tray, and leveling stick (striker) (Seedburo Equipment Co., Chicago, Il USA). During this procedure, the barley grain samples were thoroughly mixed by hand in a stainless steel pail and three 500 mL scoops were transferred into the Cox funnel which placed over the 0.5-liter cup in the tray, ensuring that the sample was mixed between each scoop. The plate was then removed from the bottom of the funnel, allowing the barley grain sample to flow freely into the cup and overflow into the tray. The Cox funnel was then carefully removed from the cup and the excess sample above the rim of the cup was removed via the leveling stick using three zig-zag motions with even pressure and consistency. The tared weight of the cup was measured and recorded (model PB3002-S, Mettler-Toledo, Inc., Lethbridge, AB Canada). Each sample was measured a total of six times and the average weight was used for calculating the PI.

Body Weight. Body weight measurements were obtained prior to the Training Period, on two consecutive days before the start of every Period, the day the animals entered and the day they left the enteric ferementation chambers, and the day the animals entered and the day they left the individual stalls during total collection for

digestibility measurements. Animals were weighed using a walk-on scale (model 29347, Mettler-Toledo, Inc., Lethbridge, AB Canada). The weights from the two consecutive days before the start of the Period were averaged together to give a single body weight for that Period.

Rumen contents. Rumen contents samples were obtained at 0, 3, 6, 9, and 12 h after feeding on d 14 and d 17 of every Period, for analysis of diurnal pH profile, dissolved H_2, biohydrogenation intermediates, protozoa, VFA, NH_3-N, microbial populations (DNA) and methyl coenzyme M reductase gene expression (RNA). All animals were fitted with a rumen canula (Extra Deep 10 cm Rumen Canula model #9CX, Bar Diamond, Inc., Parma, ID USA). A rumen fluid sample was obtained for measurement of dissolved H_2 using a homemade rumen fluid sampling filter attached to 1/8 inch tubing and a 60 mL syringe (Figure 17) which was manually inserted into the liquid phase of the rumen. A second 1 L representative sample of rumen contents (solid and liquid) was obtained from four different locations in the rumen (cranial, caudal, right, and left sides of the rumen). The sample was placed on ice and processed for analysis for remaining parameters within 15 min of sampling. During processing of each rumen contents sample, a heterogenous sample (liquid and solid combined) was retained for biohydrogenation intermediates. The remaining rumen contents sample was lightly filtered though two layers of polyester monofilament fabric (355 µm mesh opening) (PECAP) to separate the liquid and solid fractions. The lightly filtered liquid fraction was measured for pH using a stationary pH meter (SympHony model B10P, VWR North America, Radnor, PA USA) and retained for protozoa, VFA, and NH_3-N analysis. The lighly filtered rumen contents was then filtered again to further separate the remaining liquid which was retained in the solid

fraction. The liquid and solid fractions were then aliquoted into separate storage containers for microbial population (DNA) and methyl coenzyme M reductase gene expression (RNA) analysis. Total rumen digesta evaculation was also conducted to determine total rumen volume.

Diurnal pH profile. On d 14, indwelling pH meters (LRCpH Data Loggers, Dascor, Inc., Escondido, CA USA) were inserted into each animal and used to record ruminal pH at one minute intervals for a total of 7 d (3 d while in the metabolism barn and 4 d while in the enteric fermentation chambers). Meters were weighted to ensure consistent placement within the rumen and tied to the inside of the rumen cannula plug with a rope 2 ft in length. Prior to and after ruminal insertion, meters were standardized at using standard buffers (SB101-4 Buffer Solution pH 4.00 and SB107-4 Buffer Solution pH 7.00), stored in solution (BP2418-1 Electrode Storage Solution, Thermo Fisher Scientific Co., Toronto, ON Canada) and calibration values were assumed to be linear and were used to convert millivolts to ruminal pH (Penner et al., 2006). On d 21, the indwelling pH meters were removed and downloaded using data logger software (model M5-version 755 Dascor Data Logger Software, Dascor, Inc., Escondido, CA USA).

Dissolved hydrogen. Prior to measurement of dissolved H_2 using a digital microsensor amplifier with automatic sensor detection (Microsensor Multimeter version 2.01, Unisense A/S, Aarhus, Denmark), the amperometric sensor was polarized at 1,000 mV the day before sampling and allowed to acclimate for at least 8 h prior to sampling according to Guyader et al. (2015, 2017). The equipment was calibrated via an external pump (model 1001, Medical Technology Products, Inc., Huntington Station, NY USA) set at a flow rate of 499.9 mL/ h using deionized water

for the lowest concentration (0 µmol/L) and H_2 and balance CO_2 (80%, The Linde Group, Munich, Germany) bubbled in deionized water at a rate of 100 psi for the highest concentration (592.7 µmol/L) at 39 °C the day of sampling. Once the filtered rumen fluid sample was obtained, it was measured immediately for dissolved H_2 by expelling the liquid from the syringe and allowing it to pass though the sensor at a rate of approximately 10 mL of rumen fluid/ min. The electrical current was recorded every second until the graph came to a plateau according to the sensor software (SensorTrace Logger software application version 2.5.0.30462, database version 1.4, Unisense A/S, Aarhus, Denmark). At the end of the sampling period, the data were converted from electrical current (mV) to concentration (µmol/L) via the calibration curve and exported. Five consecutive data points from the plateau were averaged together for a single concentration value for each animal at each sampling time point.

Biohydrogenation intermediates. A 50 mL aliquot of rumen contents was obtained and frozen at -80 °C until fatty acid analysis. Rumen contents samples from 3 h post feeding (this was the only time point analyzed for biohydrogenation intermediates due to the cost of analysis) were thawed at room temperature (23 ± 2°C) and composited by Period and by animal. Samples were analyzed using the same procedure as TMRs as previously described.

Protozoa. A 5 mL aliquot of rumen fluid was obtained and preserved with methylgreen-formalin-saline (MFS) solution, inverted ten times, and stored in the dark at room temperature (23 ± 2°C) until analysis according to methods set forth by Ogimoto and Imai (1981). Upon enumeration and genus identification of protozoa, the sample was mixed via inversion ten times. A small sample was drawn into a 9-inch Pasteur pipet and a drop was immediately placed onto each chamber of a cell

counting chamber (Bright-Line Hemacytometer: Neubauer Improved, 0.100 mm Tiefe Depth, 0.0025 mm^2, model #62-1150, Almedic, Montreal, QC Canada). A coverslip was then placed on the counting chamber and the sample was allowed to settle for thirty to sixty seconds prior to observation under a microscope (Primo Star Halogen/ LED Microscope, model #415500-0051-000000, Carl Zeiss Canada, Ltd., North York, ON Canada). Five of the larger nine squares (starting at the upper left, the next one directly under it, and the 3 large squares on the bottom row) were used for protozoa genus identification and enumeration. Any protozoa that touched the upper and left boundaries were considered "in" and those that touched the lower and right boundaries were considered "out". Each sample was counted at least two times (i.e. count 1= chamber A, count 2= chamber B) ensuring that there was less than a ten percent difference between the two counts. If there was greater than a ten percent difference between the two counts, both chambers were counted again using new drops of the sample. Protozoa genus was determined via morphological characteristics including shape (spherical, ovoid, ellipsoidal, elongated, or asymmetrical), location of ciliary zones (absent, entire body surface, almost entire body surface, anterior and posterior body surface, or anterior body surface only), number of ciliary zones in species having them at anterior body surface only (one or two), concretion vacuole (present or absent), operculum (present or absent), skeletal plate (present or absent), number of skeletal plates (one, two, three, four, or five), shape of skeletal plates (broad or slender), number of contractile vacuoles (one, two, three, four, five, or more), shape of macronucleus (spherical, ellipsoid, rod-shaped, or more complicated), location of micronucleus (anterior, middle, or posterior part of macronucleus), and number of

caudal spines (zero, one, two, three, four, five, or more). Identification and enumeration counts were then used to calculate total genus populations.

Volatile fatty acids. A 5 mL aliquot of rumen fluid was obtained and acidified with 1 mL 25% w/v metaphosphoric acid (5:1), inverted ten times, and frozen at -20 °C until analysis. Volatile fatty acid analysis was conducted according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 285.2001.00, Title: Volatile Fatty Acids: Gas Chomatographic Analysis, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and methods of Cottyn and Boucque (1968) and Playne (1985). Rumen fluid samples were thawed and centrifuged at 14,645 × *g* for 2 min at 4 °C (Model 5415 D Eppendorf Microcentrifuge with rotor F 45-24-11, Eppendorf AG, Hamburg, Germany) and analyzed in duplicate for VFA including the major acids (acetic, propionic, and butyric) and minor acids (isobutyric, isovaleric, valeric, and capronic). Standard VFA solutions from 100 to 0 mM for acetic, propionic, and butyric acids and from 10 to 0 mM for isobutyric, isovaleric, valeric, and capronic acids (Sigma-Aldrich Canada Co., Oakville, ON Canada) (ratio of 10:1) were analyzed once at the beginning of every run for determination of concentration curve. Quality Control (QC) VFA solutions from 85 to 10 mM for acetic, propionic, and butyric acids and from 8.5 to 1.0 mM for isobutyric, isovaleric, valeric, and capronic acids (Sigma-Aldrich Canada Co., Oakville, ON Canada) (low, medium, and high concentration; ratio of 10:1) were analyzed in three sets equally spaced thoughout every run for determination of quality control. Two water blanks (HPLC Grade, Sigma-Aldrich Canada Co., Oakville, ON Canada) were analyzed immediately before and after the sets of QC samples. At the end of every run, known rumen fluid standards were

analyzed in duplicate and a VFA mixed analytical standard (Supelco 46975-U, Sigma-Aldrich Canada Co., Oakville, ON Canada) (10 mM) was analyzed. All external standards and samples contained 200 µL of 0.1 M crotonic acid (trans-2-butenoic acid) (Sigma-Aldrich Canada Co., Oakville, ON Canada) as an internal standard. Concentrations of VFA were measured using an automated gas-liquid chomatograph (Model 6890 Hewlett-Packard; Palo Alto, CA USA) with splitless injection capability. Samples were filtered though an inlet liner (4 mm with deactivated glass wool; Supelco 2-0486-05, Sigma-Aldrich Canada Co., Oakville, ON Canada) to trap any extraneous particulate matter and sampled with a 10 µL tapered syringe equipped with a polytetrafluoroethylene (PTFE)-tipped plunger (model 5181-3361, Agilent Technologies Canada Inc., Mississauga, ON Canada). Samples were analyzed with a flame-ionization detector (FID) using nitrogen (25-30 mL min^{-1}), gH_2 (29-31 mL min^{-1}), and air (250-300 mL min^{-1}) and helium (28.5 cm s^{-1}) as the carrier gas (Praxair Products Inc., Mississauga, ON Canada). Volatile fatty acids were separated on a bonded polyethylene glycol-type phase column (30 m × 0.25 mm i.d. × 1.0 µ phase thickness; Supelco Nukol 24207, Sigma-Aldrich Canada Co., Oakville, ON Canada) fitted with a polar retention gap (1.0 m × 0.25 mm i.d.; Supelco 2-5712, Sigma-Aldrich Canada Co., Oakville, ON Canada). The injector port was set at 225 °C with a split ratio of 50:1 (tolerance ± 5%) and a split flow of 1.51 mL/min of helium (valve open at 0.5 min). The column temperature was maintained at 150 °C for one min and ramped up at 5 °C min^{-1} and held at the final temperature of 195 °C for five min and the detector temperature was maintained at 250 °C. All data were analyzed using computer-controlled chromatography software (HP 3365 Series II ChemStation software, version A.03.34; Agilent Technologies Canada Inc., Mississauga, ON

Canada) and the peak area ratios, standard curves, concentrations, acetate to propionate ratio, and acetate and butyrate to propionate ratio were calculated.

All standard sample means were monitored for accuracy and precision. Standard VFA curves were monitored for linearity with $R^2 > 0.995$ for acetic, propionic, and butyric acids and $R^2 > 0.990$ for isobutyric, isovaleric, valeric, and caproic acids and any standard curve residuals >15% of theoretical values were flagged. Accuracy and precision was calculated for quality control standard sample means. Quality control and rumen fluid standard sample means were 90.0-110.0% of anticipated concentration with $\alpha < 0.10$ for acetic, propionic, and butyric acids and 85.0-115.0% of anticipated concentration with $\alpha < 0.15$ for isobutyric, isovaleric, valeric, and caproic acids and flagged if they did not meet these requirements. Volatile fatty acid mixed analytical standard was plotted against the cumulative mean (±2 standard deviations) and against a 10 mM line. Calculated VFA concentrations were 9 to 11 mM for acetic, propionic, and buyric acids (<10% error) and 8.5 to 11.5 mM for isobutyric, isovaleric, valeric, and caproic acids (<15% error) and flagged if they did not meet these requirements.

Ammonia-N. A 5 mL aliquot of rumen fluid was obtained and acidified with 1 mL 0.2 *M* sulfuric acid (5:1), inverted ten times, and frozen at -20 °C until NH_3-N analysis. Samples were analyzed for NH_3-N according to a modified version of the guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 226.2003.00, Title: Ammonia Nitrogen in 2N KCl Chernozemic Soil Extracts: Segmented Flow Analyzer, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and methods and techniques of Crooke and Simpson (1971), Dorich and Nelson (1983), Gentry and

Willis (1988), Kempers and Zweers (1986), Nelson (1983), Pym and Milham (1976), Rhine et al. (1998), Rowland (1983), Searle (1984), Searle (1990), Technicon Industrial Systems (1974), Verdouw et al. (1977), APHA et al. (1995), Garfield et al. (1991), and O'Dell (1993). This was a colorimetric assay for the determination of NH_3-N in which ammonia, sodium salicylate, sodium nitroprusside, and sodium hypochlorite form an emerald-green color (indophenol) in a buffered alkaline (pH of 12.8 to 13.0) medium at 37 ± 3.0°C and the ammonium-salicylate complete is read at 660 nm. Rumen fluid samples were thawed and centrifuged at 14,645 × *g* for 2 min at 4 °C (Model 5415 D Eppendorf Microcentrifuge with rotor F 45-24-11, Eppendorf AG, Hamburg, Germany). The average the weight of a 5 mL sample of supernatant (5 replicates) was used to calculate the dilution rate. All supernatants were diluted (1:10) using an automated diluter (model L2506 Microlab Dispenser/ Dilutor, Hamilton Co., Reno NV USA) using a 1 mL syringe set at speed 5 and programed by software (LabView software Hamilton Co., Reno NV USA) and analyzed in duplicate for NH_3-N. Standard NH_3-N solutions (1 mg/mL N or 1.22 mg/mL NH_3 or 3.816 mg/mL NH_4Cl) from 0.0 to 5.0 mg/ L of N as NH_3 (Thermo Fisher Scientific Co., Toronto, ON Canada) were analyzed once at the beginning of every run for determination of concentration curve. Quality Control (QC) NH_3-N solutions from 0.2 to 5.0 mg/ L of N as NH_3 (Thermo Fisher Scientific Co., Toronto, ON Canada) (low, medium, and high concentration) were analyzed in three sets equally spaced thoughout every run for determination of quality control. Two water rinses (HPLC Grade, Sigma-Aldrich Canada Co., Oakville, ON Canada) were analyzed immediately before and after the sets of QC samples. At the end of every run, a known soil standard (Laboratory

Control Quality Control Standard Soil #7 in 2N KCl) and a spiked sample (Analyte Recovery Sample) was also analyzed.

Samples and standards were collected with an electronic pipette (Rainin EDP, Mettler Toledo Rainin, LLC., Oakland, CA USA), which was connected to an autosampler with racks (model 311XYZ, Astoria-Pacific Inc., Clackamas, OR USA). The sample was pumped (model 133-A014-01 Technicon Proportioning Pump III, Technicon Industrial Systems. Tarrytown, NY USA) though a NH_3-N analytical cartridge in a manifold (model 170-0108-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA) with a cover (model 170-0233-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA). The sample was then analyzed on a digital detector (model 305D Astoria-Pacific Digital Detector, Astoria-Pacific Inc., Clackamas, OR USA) and recorded (FASPacII software, Astoria-Pacific Inc., Clackamas, OR USA) and the standard curves and concentrations were calculated.

Accuracy and precision were calculated for standard sample means. Quality control standard and known soil standard values were used to construct a means chart in which required linearity with $R^2 > 0.999$ and calculated upper and lower warning levels defined at ± 2 standard deviations from the mean and upper and lower control levels defined at ± 3 standard deviations from the mean and flagged if they did not meet these requirements. The standard spiked sample was used to determine the percent recovery of the assay and recovery of 95% to 105% was deemed acceptable. Microbial populations. A 30 mL liquid sample and a 30 g solid sample were retained and frozen at -80 °C until DNA analysis.

Methyl coenzyme M reductase gene expression. A 5 mL liquid sample and a 5 g solid sample were immediately flash frozen in liquid nitrogen and then frozen at -80 °C until RNA analysis.

Rumen evacuation. Total rumen digesta was manually evacuated from each cow via the ruminal canula at 1400 h 3 d after the end of the trial, 4 h postfeeding for determination of rumen volume. During evacuation, rumen digesta was empied into 55 gallon trash cans equipped with two to three 1-gallon jugs of water at 39°C and covered with a lid. A 700 to 1,000 g aliquot of rumen digesta was also obtained and dried at 50 °C for 96 h (rotated daily) in a horizontal air flow oven (VWR model #1690, Sheldon Manufacturing, Inc., Cornelius, OR USA) for determination of dry matter content. After dried samples were taken out of the oven, they were allowed to cool to room temperature before the final weights were measured.

Enteric fermentation. Enteric gas production (CH_4, CO_2, and H_2) was measured during Week 3 (d 18 to 21) of every Period at the Controlled Environment Building at Agriculture and Agri-Food Canada Lethbridge Research and Development Center in Lethbridge, Alberta according the methods and techniques of Beauchemin and McGinn (2006) and McGinn et al. (2004). Prior to animal entry on the first day of sampling and once each day of sampling, all of the chambers (#136, #140, #141, and #142) were calibrated for water vapor via a portable dew generator (model LI-610, LI-COR Environmental, Lincoln, NE USA), CH_4 gas using a known standard (40.3 ppm, Praxair Products Inc., Mississauga, ON Canada) and CO_2 using balance air (450 ppm, Praxair Products Inc., Mississauga, ON Canada). Gas tanks were equipped with regulators (Dual-Stage High-Purity Brass Regulator model 3120 series, Matheson Tri-Gas, Inc., Montgomeryville, PA USA) to control flow. The system was then flushed

with N_2 gas (Praxair Products Inc., Mississauga, ON Canada) using a regulator (ProStar Platinum High Purity Chome Regulator model PRS-3012-23, Praxair Products Inc., Mississauga, ON Canada) to ensure complete evacuation of any remaining gases used for calibration.

During sampling, each animal was restrained in a metabolism stall measuring 2.5 m long × 0.9 m wide and elevated above the floor by 15 cm within the individual climate-controlled, open circuit chamber. The chamber measured 4.4 m wide × 3.7 m deep × 3.9 m tall (63.5 m^3 volume; model C1330; Conviron Inc., Winnipeg, MB, Canada). During each Period, the same animal was housed in the same chamber to limit variability due to slight differences between chambers (thus, effect of animal and chamber was confounded). Each chamber was equipped with a radio for background noise, two large mirrors to mimic a neighboring animal, and large rubber toys to inhibit boredom. Temperature and air velocity of the fresh-air intake and exhaust ducts of each individual chamber were regulated constantly and recorded via a temperature control system with a thermocouple junction with shielded cable (Emerson Climate Technologies Computer System Process Controls, Brantford, ON Canada) and an air velocity transducer (model 8455, TSI Inc., Shoreview, MN USA). Each individual chamber was equipped with its own ventilation system including fresh-air intakes and chamber exhaust ducts with an internal diameter of 30.5 cm and fans dedicated to each duct (Figure 15). To ensure a representative air sample in the chamber exhaust ducts, air within the chamber was recycled every five min. During the air recycling process, fresh intake air was fed at a rate of 0.28 m^3/s into a sealed box containing two squirrel-type fans. The fresh intake air was then fed into the chamber via three raised floor vents located next to the animal. This air was recycled

and filtered in the vents above the stall. The temperature of the recycled air was kept consistent (at approximately 15° C) by passing through a condenser located on the low-pressure side of the recycling fans. Besides the sealed door to enter and exit the chamber, the only other location in which air had the possibility of entering the chamber was through the manure removal track located in the rear of the chamber floor. To inhibit the entrance of unwanted air from this location, a flexible rubber mat was hung vertically about the manure track, which sealed the chamber off from the manure removal system. Additionally, positive pressure of three to five Pa was consistently maintained while animals were in the chambers by differences in air flow rate between the fresh intake air duct (0.28 m^3/s) and the chamber exhaust duct (0.22 m^3/s).

Animals were fed once a day at staggered times 7 minutes apart based on gas concentration recording intervals. Therefore, the animal in chamber #136 was fed at 10:37, the animal in chamber #140 was fed at 10:44, the animal in chamber #141 was fed at 10:51, and the animal in chamber #142 was fed at 10:58 (Table 10). These were the only times during the day in which the chambers were opened (however, in the case of emergency, chambers were opened immediately regardless of the time). During feeding and every time a chamber door was opened, an access file was recorded and used during statistical analysis to remove these times from the data set. During these times, personnel removed the feed bunk with the previous day's refusals and replaced it will a feed bunk containing feed for the current day, removed any urine and feces, and obtained daily water intake recorded via flow meters (model #8188, Gardena Canada Ltd., Brampton, ON Canada). Actual time that personnel were in the chambers was limited and recorded on the individual door access sheet.

Carbon dioxide and methane. Carbon dioxide and CH_4 concentrations of the chambers were measured in rotation, using the numerical order of the chambers, every 3 minutes (Table 11). During this time, at the start of gas measurements (0 minutes of every h), chamber #136 was measured for exhaust CO_2 and intake CH_4. Then, at 3 minutes, chamber #136 was measured for intake CO_2 and exhaust CH_4. At 6 minutes, the next chamber, chamber #140, was then measured for intake CO_2 and exhaust CH_4 and then exhaust CO_2 and intake CH_4 at 9 minutes (Table 11). This rotation continued until all of the chambers were measured for intake and exhaust of both CO_2 and CH_4. After a full rotation, 3 minutes of a reference gas (N_2) was used to flush the system prior to the initiation of another round of sampling. Therefore, all chambers were sampled for intake and exhaust of both CO_2 and CH_4 twice in one h for a total of 48 times a day.

During sampling, each chamber had two tubes (one for exhaust and one for intake gas) which ran though a 0.45 µm syringe filter (model #02915-30 Cole-Parmer, Montreal, QC Canada) and back to the control room (8 tubes total) which was controlled by a datalogger (model CR23X Micrologger) with a computer (model RS-232) and a relay driver (model A6REL-12) (Campbell Scientific Inc., Logan, UT USA) using a regulated 12.5-volt DC power and a high efficiency switch mode power supply (model MW126A, Mean Well, New Taipei City, Taiwan) which was responsible for the timing and recording of the gas measurements. All of the 8 tubes coming from the chambers passed though a highly accurate, low pressure laboratory transducer (model PX653-0.1D5V, Omega Engineering Inc., Stamford, CT USA) which directed the gas towards the appropriate solenoid valve divider (Skinner model #169460, Parker Hannifin Canada Motion & Control Division, Toronto, ON Canada)

based on what gas was being sampled at that time. The sample for CO_2 concentration (ppm, volume basis) was shunted past the mass flow controller (model MC Series, Alicat Scientific, Tuscan, AZ USA) and O_2 analyzer (model FC-10, Sable Systems Int., Las Vegas, NV USA), before reaching the CO_2 analyzer (model LI-7000, LI-COR Environmental, Lincoln, NE USA), which also measured the water vapor content of the airstream to account for dilution and pressure broadening effects of H_2O and to correct for CO_2 concentration. Barometric pressure was also monitored by an external pressure transducer (model CS105 barometer, Vaisala Inc., Vantaa, France), which sent an analog signal back to the CO_2 analyzer for measurement of CO_2 and H_2O. The sample for CH_4 concentration (ppm) was shunted though the anhydrous $CaSO_4$ laboratory gas drying unit (model #26800, W. A. Hammond Drierite Co. Ltd., Xenia, OH USA), gas dryer (Nafion 144″ Dryer ¼ Poly Fit/Shell model MD-110-144P-4, Perma Pure LLC, Lakewood, NJ USA) and the $Mg(ClO_4)_2$ scrubber (Big Trap model #1/8":202260, Chromatography Research Supplies Inc., Louisville, KY USA) and eventually reaching the CH_4 analyzer (model Ultramat 6, Siemens Inc., Karlsruhe, Germany) with a 0 to 50 ppm range.

Hydrogen. Hydrogen concentration was measured simultaneously for all chambers at thirty minute intervals 3 h apart. Hydrogen collection occurred at 01:30 - 02:00, 04:30 - 05:00, 07:30 - 08:00, 10:30 - 11:00, 13:30 - 14:00, 16:30 - 17:00, 19:30 - 20:00, 22:30-23:00. It was also noted that the 10:30-11:00 sampling time point was removed from analysis as this accounted for the time in which the chamber doors were opened daily for feeding purposes. Hydrogen was collected into 15.25 × 14.75 inch 4-mm thick impermeable foil/ polymetallic bags (model S-18139, Uline Canada, St. Brampton, ON, Canada) furnished with a valve approximately 2.50 inches from the

top of the bag and housed in a 6.5-gallon pail with a lid (Lite Latch® model #665000, M & M Industries Inc., Chattanooga, TN USA) equipped with a vacuum system to facilitate filling of the bag. Hydrogen sampling was controlled by a wiring panel with display keyboard (model CR10X and CR10KD, Campbell Scientific Inc., Logan, UT USA), a relay (1-DC Series DC Output Solid State Relay Panel Mount model D1D07, Crydom, San Diego, CA USA), and a solenoid pump (Skinner model #169460, Parker Hannifin Canada Motion & Control Division, Toronto, ON Canada). Hydrogen concentration within the bag was determined via a H_2 breath tester (BreathTracker Digital Microlyzer, QuinTron Instrument Company, Inc., Milwaukee, WI USA) equipped with a pump (AlveoVac Extraction System, QuinTron Instrument Company, Inc., Milwaukee, WI USA) within 12 h of sampling. Prior to H_2 concentration analysis, the breath tester was calibrated using a combination of H_2, CH_4, and CO_2 gases (153 ppm H_2, 76 CH_4, and 6.2% CO_2, QuinGas-3 Calibration Gas, QuinTron Instrument Company, Inc., Milwaukee, WI USA).

Urine and feces. Total collection of urine (for nitrogen balance measurements including allantoin, urea, ammonia, uric acid, and nitrogen) and feces (for diet digestibility measurements) were obtained during Week 4 (d 24 to 27) of every Period. Prior to total collection of urine and feces, animals were removed from their stalls and put into individual tie stalls. No bedding (only mats) were used during the total collection to limit contamination of urine and feces with organic matter.

Urine catheters were installed in the animals one to two h before the start of urine collection according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 4210.000 Revision #004, Title: Urine Collection: Female

Catheterization (large Bovine), Agriculture and Agri-Food Canada, Lethbridge, AB Canada). Before catheterization, all animals were administered a non-steriodal anti-inflammatory injectable solution of meloxicam (Metacam 20 mg/mL, Boehinger Ingelheim Ltd., Burlington ON Canada) intravenously, at a rate of 0.5 mg/ kg of body weight, for relaxation. During the catheterization, animals were placed in a squeeze chute (Parallell Axis Cattle Squeeze Chute model # 1550, Hi-Hog Farm & Ranch Equipment, Ltd., Calgary, AB Canada) while one person restrained the tail comfortably to the side of the animal in order to limit discomfort. The rectum and vulva were washed in triplicate with an iodine (Prepodyne Scrub, West Penetone Inc., Anjou, QC Canada) water scrub and cleaned using a new paper towel each washing. The inside of the vulva was also washed in duplicate or triplicate by squirting the iodine water solution inside the vulva using a 500 cc syringe (Génia, Saint-Hilaire-de-Chaléons, France) and by carefully washing away from the vulva (inside out). The integrity of each catheter (Foley Catheter LUBRICATH® model #012375, size balloon 75cc 26 French, Bard Medical Division, C. R. Bard, Inc., Covington, GA USA) was tested by slightly opening the sterile package, injecting 10 cc of sterile saline solution (0.9% Sodium Chloride Injection USP JB 1324, Baxter Healthcare, Mississauga, ON Canada) into the balloon, observing the apparatus for any cracks or leaks, and then withdrawing the saline solution. Upon installation of the catheters, all personnel handling the catheters cleaned their hands and fingernails with iodine wash and a scrub brush to inhibit contamination of the apparatus. Using a water-based lubricant (K-Y Jelly Personal Lubricant, Johnson and Johnson, New Brunswick, NJ USA), four fingers were inserted into the vulva of the animal and the tip of the catheter was slowly directed along the top of the suburethal diverticulum and down the

external urethal orifice. After the catheter was successfully inserted into the bladder, the catheter balloon was immediately filled with 70 to 75 cc of sterile saline solution, allowing the animal to urinate as normal without expelling the catheter. As a precautionary measure to limit the possibility of a bladder infection, immediately after the catheter was inserted and the balloon filled, an antibiotic (Trimidox model #25739, Vétoquinol N.-A. Inc., Lavaltrie, QC Canada) was administered at a rate of 3 mL/ kg of body weight, directly though the catheter and into the bladder. The end of the catheter was clamped for ten to fifteen minutes, to allow the antibiotic to flush the bladder, before the clamp was removed, allowing the animal excrete the antibiotic. Tubing (Nalgene 180 PVC Tubing model #8000-0070, Nalge Nunc International Corporations, Rochester, NY USA) was then inserted into the open end of the catheter (at least 2 cm deep) and secured in place via a cable tie while the other end of the tubing was secured in a 20 L collection vessel. An injection of meloxicam was also administered subcutaneously every forty-eight h after the catheters were inserted. The animals were monitored multiple times daily and fecal matter was removed from the tubing and vulva and the area was cleaned using a towel and iodine water solution at least once a day. The health status of the animals and minor urine parameters was also monitored daily by testing fresh urine for specific gravity (1.000 – 1.030), pH (5 – 9), the presence of leukocytes (0 – 500 leukocytes / µL), nitrite (negative / positive), protein (0 – 500 mg/dL), glucose (0 – 1,000 mg/dL), ketones (0 – 150 mg/dL), urobilinogen (0 - 12 mg/dL), bilirubin (0 – 6 mg/dL), and blood erythocytes (0 – 250 erythocytes / µL); using urine reagent test strips (Chemstrips® 10 MD Urine Test Strips, Roche Diagnostics Corporation, Indianapolis, IN USA) according to manufacturer's guidelines (Roche Diagnostics Corporation, 2010a) and methods and

techniques of Roche Diagnostics Corporation (2010b,c), Gambke et al. (1994), Gambke et al. (1997), and Gambke (1995).

Urine was collected into a 25-L collection jug containing 500 – 1,300 mL of 4 N sulfuric acid to keep the urine at a pH < 3 in order to inhibit microbial degradation of components. To ensure that the pH of the urine was consistently below pH 3, the urine in the collection jug was tested every 24 h using pH test strips (model BDH35310.601, VWR North America, Radnor, PA USA). If the pH of the urine increased to above pH 3.0, more acid was added. After every 24 h interval, the total volume of urine was carefully poured though a funnel and into a 10-L Plexiglas marked container and the volume was recorded. A 200 mL representative subsample of acidified urine was removed from the collection jug and filtered though four layers of cheesecloth. Twenty mL of acidified urine was retained as a back-up sample and frozen at -20 °C. Another 15 mL of acidified urine was diluted with 60 mL of water (to inhibit precipitation of components) for a dilution of 1:5. The diluted sample was mixed thoroughly and frozen at -20 °C until analysis.

Feces was collected in total collection tubs and every 24 h during the total collection sampling, the feces was thoroughly mixed. At this time, a 30 g aliquot was mixed with 120 mL of deionized water, shaken for 15 min at 100 × *g* (Junior Orbit Shaker, Lab-Line Instruments, Inc., Melrose Park, Il USA), and the pH was determined (SympHony model B10P, VWR North America, Radnor, PA USA). A 700 to 1,000 g aliquot of feces was also obtained and dried at 50 °C for 96 h (rotated daily) in a horizontal air flow oven (VWR model #1690, Sheldon Manufacturing, Inc., Cornelius, OR USA) for determination of dry matter content. After dried samples were taken out of the oven, they were allowed to cool to room temperature before the final weights

were measured. All dried feces samples were stored at room temperature and retained for chemical analysis.

During urine analysis, samples were thawed at room temperature and samples were divided into multiple 2-mL centrifuge tubes in which one was retained as a back-up sample and the remaining were analyzed for purine derivatives (allantoin and uric acid), urea, ammonia, and nitrogen.

Allantoin. A 1 mL diluted acidified urine sample was further diluted 5x for a final dilution rate of 1:10 and analyzed for allantoin according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Title: Allantoin: A colorimetric method, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and by methods of Young and Conway (1942) and Chen and Gomes (1992). Diluted urine samples were analyzed in duplicate for allantoin concentration. Standard allantoin solutions with 10 ± 0.05, 20 ± 0.05, 30 ± 0.05, 40 ± 0.05, 50 ± 0.05, 60 ± 0.05, 80 ± 0.05 and 100 ± 0.05 mg of allantoin/L were analyzed once at the beginning of every run for determination of concentration curve. A water blank was also analyzed in every run for determination of quality control. Water blank, standards, and samples were transferred to cuvettes and absorbency was read at 522 nm using a spectrometer (ThermoSpectronic Genesys 20 Visible Spectrophotometer, Thermo Fisher Scientific Co., Toronto, ON Canada). Any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run.

Uric acid. Samples were analyzed for uric acid according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (Title: Uric Acid Analysis, Agriculture

and Agri-Food Canada, Lethbridge, AB Canada) and methods and techniques of Folin and Dennis, 1913; Caraway, 1963; Morin and Prox, 1973; Morin, 1974; Brochner-Mortensen, 1940; Klacker, 1947; Praetorius and Poulsen, 1953; NCCLS, 1991a; NCCLS, 1991b, NCCLS, 1992; Henry et al., 1974; and Young et al., 1975. Urine samples were thawed and centrifuged at 14,645 × *g* for 2 min at 4 °C (Model 5415 D Eppendorf Microcentrifuge with rotor F 45-24-11, Eppendorf AG, Hamburg, Germany). All supernatants were analyzed in duplicate for uric acid. Standard uric acid solutions from 0.0 to 5.0 mg/ dL of uric acid (CAS # 69-93-2, Sigma-Aldrich Canada Co., Oakville, ON Canada) were analyzed once at the beginning of every run for determination of concentration curve. Quality Control (QC) uric acid samples with known concentrations of uric acid (Level I Chemistry Control (4.7 ± 0.8 mg/dL) and Level II Chemistry Control (9.0 ± 1.5 mg/dL) of human serum with enzymes, non-protein constituents, non-human protein, and bacteriostatic agents that were adjusted to meet expected values; Pointe Scientific, Canton, MI USA) were analyzed thoughout every run for determination of quality control. Standards and samples were measured at 508 nm using a microplate reader (Dynatech MRX Microplate Reader, Dynatech Laboratories Inc., Alexandria, VA USA) according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 3130.000 Title: Microplate Reader: Operation of Dynatech MRX, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and uric acid concentration was calculated.

Urea. Samples were analyzed for urea according to guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (Title: Astoria2 Analyzer Urea Analysis,

Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and methods and techniques of Rahmatullah and Boyde (1980). Urine samples were thawed and centrifuged at 14,645 × g for 2 min (Model 5415 D Eppendorf Microcentrifuge with rotor F 45-24-11, Eppendorf AG, Hamburg, Germany). All supernatants were diluted (1:100) using an automated diluter (model L2506 Microlab Dispenser/ Dilutor, Hamilton Co., Reno NV USA) and analyzed in duplicate for urea. Standard urea solutions from 0.50 to 200.0 mg/ L of urea (CAS # 57-13-6, Sigma-Aldrich Canada Co., Oakville, ON Canada) were analyzed once at the beginning of every run for determination of concentration curve. Quality Control (QC) urea samples with known concentrations of urea (Level I Chemistry Control (14 ± 2 mg of N/ L) and Level II Chemistry Control (51 ± 5 mg of N/ L) of human serum with enzymes, non-protein constituents, non-human protein, and bacteriostatic agents which were adjusted to meet expected values; Pointe Scientific, Canton, MI USA) were analyzed thoughout every run for determination of quality control. A water rinse (HPLC Grade, Sigma-Aldrich Canada Co., Oakville, ON Canada) was analyzed immediately before and after the calibration curve and QC samples. Samples and standards were collected with an electronic pipette (Rainin EDP, Mettler Toledo Rainin, LLC., Oakland, CA USA) connected to an autosampler with racks (model 311XYZ, Astoria-Pacific Inc., Clackamas, OR USA). The sample was pumped (model 133-A014-01 Technicon Proportioning Pump III, Technicon Industrial Systems. Tarrytown, NY USA) though a urea analytical cartridge in a manifold (model 170-0108-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA) with a cover (model 170-0233-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA). The sample was then analyzed on a digital detector (model 305D Astoria-

Pacific Digital Detector, Astoria-Pacific Inc., Clackamas, OR USA) and recorded (FASPacII software, Astoria-Pacific Inc., Clackamas, OR USA) and the standard curves and concentrations were calculated.

Ammonia-N. Samples were analyzed for NH_3-N according to a modified version of the guidelines set forth by the Livestock Sciences Section (Sustainable Production Systems) of the Lethbridge Research and Development Centre (SOP Code: 226.2003.00, Title: Ammonia Nitrogen in 2N KCl Chernozemic Soil Extracts: Segmented Flow Analyzer, Agriculture and Agri-Food Canada, Lethbridge, AB Canada) and methods and techniques of Crooke and Simpson (1971), Dorich and Nelson (1983), Gentry and Willis (1988), Kempers and Zweers (1986), Nelson (1983), Pym and Milham (1976), Rhine et al. (1998), Rowland (1983), Searle (1984), Searle (1990), Technicon Industrial Systems (1974), Verdouw et al. (1977), APHA et al. (1995), Garfield et al. (1991), and O'Dell (1993). This was a colorimetric assay for the determination of NH_3-N in which ammonia, sodium salicylate, sodium nitroprusside, and sodium hypochlorite form an emerald-green color (indophenol) in a buffered alkaline (pH of 12.8 to 13.0) medium at 37 ± 3.0 °C and the ammonium-salicylate complete is read at 660 nm. Urine samples were thawed and centrifuged at 14,645 × *g* for 2 min at 4 °C (Model 5415 D Eppendorf Microcentrifuge with rotor F 45-24-11, Eppendorf AG, Hamburg, Germany). All supernatants were diluted (1:15) using an automated diluter (model L2506 Microlab Dispenser/ Dilutor, Hamilton Co., Reno NV USA) using a 1 mL syringe set at speed 5 and programed by software (LabView software Hamilton Co., Reno NV USA) and analyzed in duplicate for NH_3-N. Standard NH_3-N solutions (1 mg/mL N or 1.22 mg/mL NH_3 or 3.816 mg/mL NH_4Cl) from 0.0 to 5.0 mg/ L of N as NH_3 (Thermo Fisher Scientific Co., Toronto,

ON Canada) were analyzed once at the beginning of every run for determination of concentration curve. Quality Control (QC) NH_3-N solutions from 0.2 to 5.0 mg/ L of N as NH_3 (Thermo Fisher Scientific Co., Toronto, ON Canada) (low, medium, and high concentration) were analyzed in three sets equally spaced throughout every run for determination of quality control. Two water rinses (HPLC Grade, Sigma-Aldrich Canada Co., Oakville, ON Canada) were analyzed immediately before and after the sets of QC samples. At the end of every run, a known soil standard (Laboratory Control Quality Control Standard Soil #7 in 2N KCl) and a spiked sample (Analyte Recovery Sample) was also analyzed.

Samples and standards were collected with an electronic pipette (Rainin EDP, Mettler Toledo Rainin, LLC., Oakland, CA USA) which was connected to an autosampler with racks (model 311XYZ, Astoria-Pacific Inc., Clackamas, OR USA). The sample was pumped (model 133-A014-01 Technicon Proportioning Pump III, Technicon Industrial Systems. Tarrytown, NY USA) though a NH3-N analytical cartridge in a manifold (model 170-0108-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA) with a cover (model 170-0233-01 Technicon Autoanalyzer II, Technicon Industrial Systems. Tarrytown, NY USA). The sample was then analyzed on a digital detector (model 305D Astoria-Pacific Digital Detector, Astoria-Pacific Inc., Clackamas, OR USA) and recorded (FASPacII software, Astoria-Pacific Inc., Clackamas, OR USA) and the standard curves and concentrations were calculated.

Accuracy and precision were calculated for standard sample means. Quality control standard and known soil standard values were used to construct a means chart in which required linearity with $R2 > 0.999$ and calculated upper and lower warning

levels defined at ± 2 standard deviations from the mean and upper and lower control levels defined at ± 3 standard deviations from the mean and flagged if they did not meet these requirements. The standard spiked sample was used to determine the percent recovery of the assay and recovery of 95% to 105% was deemed acceptable.

Nitrogen. Samples were analyzed for N content according to the Dumas method of AOAC International (1995) (AOAC Official Method 968.06). Urine samples were analyzed in duplicate for nitrogen content by measuring out 50 µL of sample into 8 × 5 mm smooth wall tin capsules (D4057, Elemental Microanalysis Ltd., Okehampton, UK). Samples were dried at 55 °C for 24 h prior to analysis. Standard nitrogen samples with 1 ± 0.05, 3 ± 0.05, 5 ± 0.05, and 8 ± 0.05 mg of soft spring wheat standard (B.M.O.) were analyzed every twenty-four samples for determination of concentration curve. A reference nitrogen sample with 5 ± 0.05 mg of soft spring wheat standard (B.M.O.) of known nitrogen concentration was analyzed every twelve samples for determination of quality control and any duplicate values for a sample exceeding a critical value of ± 5.0% were re-run. Samples were analyzed using an elemental analyzer (NA 2100 Elemental Analyzer, Carlo Erba Instruments, Milan, Italy) which combusted the samples at 1020 °C and the gases were then reduced to N_2 in the presence of Cu at 750 °C. Nitrogen was separated using a stainless steel porous polymer chromatography column (3 m × 6 mm × 5, Poropak QS, mm Agilent Technologies Canada Inc., Mississauga, ON Canada) using CO_2 as the carrier gas and detected using a terminal conductivity detector. Crude protein was calculated from nitrogen concentration values.

Chemical analysis. All dried samples of feces were ground to 4 mm in size (SM 100, Retsch Inc., Haan, Germany). The 4 mm ground sample was then split in two and one

sample was retained and the other was ground to 1 mm in size and used for chemical analysis using methods previously described for feed analysis for DM, OM, NDF, ADF, CP, and starch. However, the sample size for starch analysis was 500 ± 0.05 mg of feces. Total tract digestibility (percent of nutrient digested) for individual nutrients was calculated.

Calculations and Statistical Analysis**.**

To calculate NE_m intake, the following equations were used:

$$SBW = 0.96 \times \ full\ weight$$

$$EBW = 0.891 \times SBW$$

$$NE_m = 0.077 \times \ EBW^{0.75}$$

$$NE_m\ intake = \frac{NE_m}{energy\ density\ of\ diet}$$

where *SBW* is shunk body weight (kg), *EBW* is empty body weight (kg), NE_m is net energy required for maintenance (Mcal/d), NE_m *intake* is the amount of feed needed for maintenance per day based on the energy density of the diet (kg of DMI/d) (NRC, 1996).

To calculate total digestible nutrients, the following equation was used:

$$TDN = 95.88 - (0.911 \times ADF)$$

where *TDN* is total digestible nutrients (% DM) and *ADF* is acid detergent fiber (% DM, CVAS, 2009).

To calculate the bushel weight of barley grain samples, the following equation was used:

$$BU = \left(\frac{W \times L \ \times B}{P}\right)$$

where *BU* is the bushel weight (lbs/bushel) of barley grain, *W* is the average weight of the 0.5-liter cup (g), *L* is the factor needed to give the weight of a 1-liter cup

(2), *B* is the number of liters in a U.S. bushel (35.24 L/ BU), and *P* is the number of grams in a pound (454 g/lb).

To calculate the processing index of barley grain samples, the following equation was used:

$$PI = \left(\frac{AP}{BP}\right) \times 100$$

where *PI* is the processing index of barley (%), *AP* is the bushel weight of barley after processing (lbs/bushel), and *BP* is the bushel weight of barley before processing (lbs/bushel) (Rode and Beauchemin, 1998).

To calculate the analytical DM of the feed ingredients, diets, and feces; the following equation was used:

$$DM_a = \frac{A - B}{C}$$

where DM_a is the analytical dry matter (%), *A* is the crucible and dry sample weight (g), *B* is the crucible weight (g), and *C* is the original sample weight (g).

To calculate the on-farm DM of the feed ingredients, diets, and feces; the following equation was used:

$$DM_f = \frac{A - B}{C}$$

where DM_f is the on-farm dry matter (%), *A* is the bag and dry sample weight (g), *B* is the bag weight (g), and *C* is the original sample weight (g).

To calculate the correction equation for on-farm DM and analytical DM of the feed ingredients, diets, and feces; the following equation was used:

$$DM_c = \left(\frac{A}{B}\right) \times 100$$

where DM_c is the corrected dry matter (%), *A* is the on-farm dry matter using a 60° C oven for 7 d (%) and *B* is the analytical dry matter using a 135° C oven for 2 h (%).

To calculate analytical OM of the feed ingredients, diets, and feces; the following equation was used:

$$OM = \frac{A - B}{C - D}$$

where *OM* is the analytical organic matter (%, DM basis), *A* is the crucible and ashed sample weight (g), *B* is the crucible weight (g), and *C* is the crucible and dry sample weight (g), and *D* is the crucible sample weight (g).

To calculate NDF of the feed ingredients, diets, and feces; the following equation was used:

$$NDF = \frac{(100 \times (W_3 - (W_1 \times C_1))}{(W_2 \times DM_a)}$$

where *NDF* is neutral detergent fiber (%, DM basis), W_1 is the bag tare weight (g), W_2 is the sample weight (g), W_3 is the dried weight of the bag with fiber after the extraction process (g), C_1 is the blank bag correction (running average of final oven-dried weight divided by the original blank bag weight), and DM_a is the analytical dry matter (%).

To calculate ADF of the feed ingredients, diets, and feces; the following equation was used:

$$ADF = \frac{(100 \times (W_3 - (W_1 \times C_1))}{(W_2 \times DM_a)}$$

where *ADF* is acid detergent fiber (%, DM basis), W_1 is the bag tare weight (g), W_2 is the sample weight (g), W_3 is the dried weight of the bag with fiber after the extraction process (g), C_1 is the blank bag correction (running average of final oven-dried weight divided by the original blank bag weight), and DM_a is the analytical dry matter (%).

To calculate starch content of the feed ingredients, diets, and feces; the following equation was used:

$$S = \frac{G}{1.11 \times WT \times DM} \times 100$$

where *S* is starch content (%, DM basis), *G* is the amount of glucose in the sample (mg), 1.11 is the amount of glucose to yield 1 gram of starch (g), *WT* is the sample weight (mg), and *DM* is the dry matter of the sample (%).

To calculate crude protein content of the feed ingredients, diets, and feces; the following equation was used:

$$CP = N \times 6.25$$

where *CP* is crude protein content (%, DM basis), *N* is the amount of nitrogen in the sample (%), 6.25 is the standard factor which assumes that animal feeds contain approximately 16 g of nitrogen per 100 g of protein (NRC, 2001).

To calculate crude fat of the feed ingredients and diets; the following equation was used:

$$CF = \left(\frac{W_3 - W_2}{W_1 \times DM_a}\right) \times 100$$

where *CF* is the crude fat concentration (%, DM basis), W_1 is the initial sample weight (g), W_2 is tare weight of the beaker (g), W_3 is the weight of the beaker and fat residue (g), and DM_a is the analytical dry matter using a 135° C oven for 2 h (%).

To calculate gross energy of the diets and feces; the following equation was used:

$$GE = (E \times F) - (C \times CE) - \left(\frac{T}{S \times DM}\right)$$

where *GE* is the gross energy content of the sample (MJ/kg), *E* is the total energy content (MJ/kg), *F* is the total capsule and sample weight (g), *C* is the gel capsule weight (g), *CE* is the energy content of the empty gel capsule weight (MJ/kg), *S* is the sample weight (g), and *DM* is the analytical dry matter using a 135° C oven for 2 h (%).

To correct the gross energy of the diets and feces to account for the calibration of benzoic acid; the following equation was used:

$$GE_c = (GE \times (C \div E) \times 1000) \div 1000000$$

where GE_c is the corrected gross energy content of the sample (Mcal/kg), *GE* is the gross energy content of the sample (MJ/kg), *C* is the caloric value of benzoic acid (6,318 cal/g), and *E* is the energy content of benzoic acid (26.452 MJ/kg).

To calculate digestible energy of the diets; the following equation was used:

$$DE = \frac{GE_d - GE_f}{DMI}$$

where *DE* is the digestible energy content of the sample (Mcal/kg), GE_d is the total energy content of the diet (Mcal/kg), GE_f is the total energy content of the feces (Mcal/kg), and *DMI* is dry matter intake (kg/d).

To calculate gross energy intake, the following equation was used:

$$GE_{intake} = GE \times DMI$$

where GE_{intake} is the gross energy intake (Mcal/d), *GE* is the gross (total) energy content of the diet (Mcal/ kg of DMI), and *DMI* is the dry matter intake (kg/d).

To calculate digestible energy intake, the following equation was used:

$$DE_{intake} = DE \times DMI$$

where DE_{intake} is the digestible energy intake (Mcal/d), *DE* is the digestible energy content of the diet (Mcal/ kg of DMI), and *DMI* is the dry matter intake (kg/d).

To calculate the percent of nutrient digested, the following equation was used:

$$\%\, ND = \frac{NC - \mathrm{NF}}{NC} \times 100$$

where *ND* is nutrient digestion (%), *NC* is nutrient consumed (kg), and *NF* is nutrient in feces (kg) (Fahey et al., 1994; Schneider and Flatt, 1975).

To calculate ruminal pH, the following equation was used:

$$Y = mx + b$$

where *Y* is pH (dependent variable), *m* is slope (Δy/Δx), *x* is voltage (independent variable), and *b* is the y-intercept.

To calculate ruminal pH range, the following equation was used:

$$pH_{range} = pH_{max} - pH_{min}$$

where pH_{ange} is the range of ruminal pH, pH_{max} is the maximum ruminal pH, and pH_{min} is the minimal ruminal pH.

To calculate protozoa genus populations, the following equation was used:

$$P = \frac{AC \times DF \times 10{,}000}{S}$$

where *P* is the number of protozoa/ mL of rumen fluid, *AC* is the average count, *DF* is the dilution factor (2), and *S* is the number of squares counted (5).

To calculate accuracy and precision of the QC standard sample means, the following equations were used:

$$Accuracy = \frac{measured\ concentration}{claimed\ concentration} \times 100$$

$$Precision = \frac{standard\ deviation}{measured\ mean}$$

To calculate VFA peak area ratios, the following equation was used:

$$PAR = \frac{SPA}{ISPA}$$

where *PAR* is the peak area ratio, *SPA* is the sample peak area for each acid, and *ISPA* is the internal standard (crotonic acid) peak area.

To calculate VFA molar concentration, the following equation was used:

$$VFA_c = \frac{Y - b}{m}$$

where VFA_c is the molar concentration of the acid (m*M*), *Y* is peak area ratio, *b* is the y-intercept, and *m* is slope (Δy/Δx).

To calculate VFA molar proportion, the following equation was used:

$$VFA_P = \frac{VFA_i}{VFA_t} \times 100$$

where VFA_p is the molar proportion of the acid (mol/ 100 mol), VFA_i is the molar concentration of the individual acid (m*M*), and VFA_t is the molar concentration of the total VFA (m*M*).

To calculate the acetate to propionate ratio, the following equation was used:

$$AP = \frac{A}{P}$$

where *AP* is the ration of acetate to propionate, *A* is the acetate (m*M* or mol/ 100 mol), and *P* is the propionate (m*M* or mol/ 100 mol).

To calculate the acetate and butyrate to propionate ratio, the following equation was used:

$$ABP = \frac{(A + B)}{P}$$

where *ABP* is the ration of acetate and butyrate to propionate, *A* is the acetate (m*M* mol/ 100 mol), *B* is the butyrate (m*M* mol/ 100 mol), and *P* is the propionate (m*M* mol/ 100 mol).

To calculate the 1:10 dilution factor of rumen fluid supernatant for NH_3-N analysis, the following equation was used:

$$DF_R = \frac{a + b}{(a + b) - (a - 0.5)}$$

where DF_R is the dilution factor, *a* is the weight of the cup (mg), and *b* is the weight of the 5 mL supernatant (mg).

To calculate NH_3-N analyte recovery, the following equation was used:

$$Recovery = \frac{a - b}{c} \times 100$$

where *Recovery* is the NH_3-N analyte recovery (%), *a* is the measured spiked sample concentration (mg/L of N as NH_3), *b* is the measured sample concentration

(mg/L of N as NH_3), and *c* is the original spiked sample concentration (mg/L of N as NH_3).

To calculate the concentration of NH_3 in rumen fluid and urine, the following equation was used:

$$C = \left[\left(\frac{a}{10}\right) \times DF\right] \times \left(\frac{b}{c}\right)$$

where *C* is the concentration of NH_3 (m*M*), *a* is the yield of NH_3 (mg/L), *DF* is the dilution factor, *b* is the concentration of NH_3 used in the standard stock solution (g/L), and *c* is the molar mass of NH_3 (g/mol).

To calculate the dilution factor of urine to account for the acid added, the following equation was used:

$$DF_U = \frac{V_a}{(V_a - a)} \times Y$$

where DF_U is the dilution factor for urine, V_a is the total volume of urine including acid (mL), *a* is the volume of acid (mL), and *Y* is the urine parameter of interest.

To calculate allantoin in urine, the following equation was used:

$$Y = \frac{x - b}{m} \times 10$$

where *Y* is allantoin (dependent variable) (mg/L), *x* is the optical density (independent variable), *b* is the y-intercept, *m* is slope ($\Delta y/\Delta x$), and 10 is the dilution factor.

To calculate the uric acid in urine, the following equation was used:

$$C = \frac{S}{(S + R)} \times 5$$

where *C* is the concentration of uric acid (mg/dL), *S* is the standard volume (µL), and *R* is the reagent volume (µL).

To calculate the gas emission generated for each enteric fermentation chamber, the following equation was used:

$$F_{gas} = \left[C_e MW \frac{P}{RT} V_e \frac{T}{P} A\right] - \left[C_i MW \frac{P}{RT} V_i \frac{T}{P} A\right]$$

where F_{gas} is the gas emission (g/s), *C* is the concentration of gas for chamber exhaust ($_e$) and fresh air intake ($_i$) (ppm), *MW* is the molecular weight of the gas (16.04 g/mol for CH_4, 44.01 g/mol for CO_2, 32.00 g/mol for O_2, and 2.02 g/mol for H_2), P is the barometric pressure (Pa), R is the universal gas constant (8.31 J·mol-1·deg K-1), T is the stream air temperature (°K), *V* is air velocity for chamber exhaust (*e*) and fresh air intake (*i*) (m/s), and A is the cross sectional area of the air duct (0.146 m^2) (McGinn et al., 2004).

To calculate CH_4 and H_2 emissions as a proportion of GE intake, the following equation was used:

$$Gas_{GEintake} = \left(\frac{Gas_{yeild}}{MW}\right) \times E \times \left(\frac{kcal}{4.184}\right) \times \left(\frac{Mcal}{1{,}000}\right)$$

where $Gas_{GEintake}$ is the gas emission as a proportion of GE intake (%), Gas_{yield} is the amount of gas produced (g/d), *MW* is the molecular weight of the gas (16.04 g/mol for CH_4 and 2.02 g/mol for H_2), and *E* is the energy content of the gas (802 kJ/mol for CH_4 and 436 kJ/mol for H_2).

To calculate the amount of NOP consumed by the heifers, the following equation was used:

$$NOP_{intake} = DMI_c \times NOP$$

where NOP_{intake} is the amount of NOP consumed by the heifers (mg/d), DMI_c is the calculated dry matter intake of the heifers to be fed at maintenance according to NRC (1996) guidelines (7.60 kg/d), and *NOP* is the inclusion rate of NOP (200 mg of NOP/ kg of DMI).

To calculate rumen volume, the following equations were used:

$$R_S = R_C \times R_{DM}$$

$$R_L = R_C - R_S$$

where R_S is the solid fraction of the rumen contents (kg), R_C is the total weight of the rumen contents (kg), R_{DM} is the dry matter of the rumen contents (%), and R_L is the liquid fraction of the rumen contents (L).

To calculate the molar concentration of NOP in the rumen, the following equation was used:

$$NOP_{rumen} = \left(\frac{NOP_{intake}}{R_L}\right) \times \left(\frac{g}{1{,}000\ mg}\right) \times \left(\frac{mole\ of\ NOP}{121.09\ g}\right) \times \left(\frac{1{,}000\ mmol}{mole}\right)$$

where NOP_{rumen} is the molar concentration of NOP in the rumen (mmol/L or m*M*), NOP_{intake} is the amount of NOP that was consumed in a day (1,520 mg/d), and R_L is the liquid fraction of the rumen contents (L).

To calculate the amount of NDF digested by rumen protozoa, the following equation was used:

$$NDF_{protozoa} = NDF_{intake} \times ATTD \times 0.25$$

where $NDF_{protozoa}$ is the amount of NDF that is digested by protozoa (kg/d), NDF_{intake} is the amount of NDF consumed (kg/d), *ATTD* is the apparent total tract digestibility (%), and *0.25* is the amount of fiber digestion that protozoa are assumed to account for (%) (Lee et al., 2000).

To calculate the difference in H_2 production between the experimental treatments in reference to the CON, the following equation was used:

$$H_2 = (CH_{4control} - CH_{4treatment}) \times \left(\frac{mole\ of\ CH_4}{16.04\ g}\right) \times \left(\frac{4.04\ g\ H_2}{mole\ of\ CH_4}\right) - H_{2gas}$$

where H_2 is the amount of H_2 (g), $CH_{4control}$ is the CH_4 production from the animals on the CON treatment (g/d), $CH_{4treatment}$ is the CH_4 production from the animals on the

experimental treatments (OIL, NOP, or NOP+OIL) (g/d), and H_{2gas} is the dihydrogen gas production from the animals on the respective experimental treatments (OIL, NOP, or NOP+OIL) (g/d).

Normality of distribution was determined using the UNIVARIATE procedure of SAS (SAS Institute, Inc., 2015). To test for normality, the following goodness-of-fit tests were used: Shapiro-Wilk test, Kolmogorov-Smirnov test, Anderson-Darling test, and Cramér-von Mises test. Homogeneity of variance was determined using the GLM procedure of SAS (SAS Institute, Inc., 2015). To test for homogeneity, the Levene's test was used (Levene, 1960; SAS Institute, Inc., 2009).

In this experiment, the majority of the data were normally distributed and similar in variance; however, the data that did not follow a normal distribution were cleaned by determining and removing measurement errors, data entry errors, and extreme outliers, which lead to more normally distributed data (Judd and McClelland. 1989; Orr et al., 1991; Osborne, 2002). Protozoa populations were not normally distributed; therefore, a $\log_{10}$ transformation was applied prior to analysis with the arithmetic least square mean reported. Fat concentration and fatty acid profile of the diets and biohydrogenation intermediates of the rumen contents exhibited a bimodal distribution due to the addition of canola oil at 5% of DM for the OIL and NOP+OIL treatments. Therefore, the OIL- and non-OIL-containing treatments were analyzed separately for normality of distribution for these variables. This separation of treatments for these variables was deemed the more appropriate option as opposed to transforming the data due to the specific nature of the variables (which were expected to be different and assuming otherwise would cause bias) and if the data were transformed, then it would inhibit comparison to non-transformed data (Chen, 2012;

LaLonde, 2012). Skewness and kurtosis values ranging from -2 and +2 were considered acceptable in order to prove that the data were normally distributed (Trochim, 2000; George and Mallery, 2010). The central limit theorem was also employed, which demonstrated that within a sample size of at least 30 experimental units, the distribution of the mean will be normal, regardless of the distribution of the original population (Larsen and Farber, 2015). This was deemed as an appropriate assumption due to the fact that sample size was greater than 30 (8 heifers × 4 treatments= 32 experimental units) and it represented the distribution of a normal population. Although some of the variables in this experiment were separated based on OIL inclusion to determine if the data were normally distributed, all of the treatments were analyzed together. This was due to the fact that the need for stratification was not determined until after the samples were collected and the experiment was originally designed as a randomized block and not designed to analyzed the main effect of OIL (factorial). Therefore, only the overall effect of treatment was observed which not only maintained the degrees of freedom but was deemed acceptable in an experiment such as this where we anticipated differences between treatments and treatment was the single factor of primary interest (Dodge, 2008).

Data were analyzed using the MIXED procedure of SAS (SAS Institute, Inc., 2015) according to the model:

$$Y_{ij(k)} = \mu + P_i + A_j + B_{(k)} + T_{(k)} + H_j + e_{ijk}$$

where $Y_{ij(k)}$ is the dependent variable and represents the observation on animal j given treatment k at period i, μ is the overall mean, P_i is the fixed effect of period (Period 1, 2, 3, or 4), A_j is the random effect of animal, $B_{(k)}$ is the fixed effect of block

(Group 1 or 2), $T_{(k)}$ is the fixed effect of treatment, H_j is the repeated measure of time (rumen fermentation, 0, 3, 6, 9, and 12 h after feeding; diurnal pH; and gas emission data) and $e_{ij(k)}$ is the residual error. The pH data were averaged by d and by 15 min intervals and analyzed for mean, minimum, maximum, and range (maximum-minimum) of pH. The gas production measurements were determined for each day and expressed relative to DMI and GE intake of that day. The variance components were estimated using the Restricted Maximum Likelihood (REML) method and degrees of freedom were adjusted using the Kenward–Roger option. The covariance structures (compound symmetry (CS), autoregressive(1) (AR(1)), Toeplitz (TOEP), variance components (VC), unstructured (UN), heterogeneous autoregressive(1) (ARH(1)), heterogeneous compound symmetry (CSH), and heterogeneous Toeplitz (TOEPH)) were selected for the best fit based on the lowest Akaike and Bayesian information criteria values according to Wolfinger (1993) and Littell et al. (2006). Means were compared using the Tukey-Kramer honestly significant difference (HSD) test (Tukey, 1953 and Kramer, 1956) and pairwise comparisons were assigned letters using PDMIX800 Macro (Saxton, 1998). Data were reported as least squares means except for chemical composition and physical characteristics of barley silage, dry-rolled barley grain, treatment mixes, and supplement mixes, which were reported as means ± standard deviation. Statistical significance and tendencies were declared at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively and the largest SEM was reported.

Results

Animals and Diets. Overall, there were no health problems noted during the course of the study. All animals behaved normally regardless of their housing location (Metabolism Barn or Controlled Environment Building).

The cost of basal diet was CAD 1.43/ animal/ d or USD 1.00/ animal/ d (at an exchange rate of 0.7017). The barley silage cost per tonne was calculated using a multiplier of 12 times the barley grain price per bushel (21.77 kg) (Nibourg, 2015). The grain and supplement costs were calculated from actual feed ingredient market prices obtained from the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre Feed Mill (Lethbridge, Alberta Canada) in January 2016. The author would like to note that this analysis does not include the cost of the supplemented canola oil (5% of diet DM; CAD 0.48/ animal/ d or USD 0.34/ animal/ d) or the Placebo or NOP mix (200 mg/ kg of diet DM; not commercially available).

The basal diet was formulated to contain 40.48% DM, 79.72% OM, 13.07% CP, 2.99% fat (ether extract), 53.11% NDF, 38.12% ADF, 16.54% starch, and 61.15% TDN (Table 9). The actual analyzed chemical composition of the diets contained greater amounts of OM, starch, and TDN (91.30, 24.52, and 72.96%, respectively), lesser amounts of DM, NDF, and ADF (35.03, 42.67, and 25.16%, respectively), and similar (<1 standard deviation) amounts of CP (12.99%) (Table 12). The actual analyzed fat (ether extract) of the non-oil containing diets (CON and NOP; 3.16%) was similar (<1 standard deviation) to the formulated basal diet (2.99%) and the actual analyzed fat (ether extract) of the oil-containing diets (OIL and NOP+OIL; 8.40%) was considerably greater than the formulated basal diet due to the inclusion of canola oil at 5.0% of diet DM. Although the differences in formulated and actual chemical

composition of the diets were small (<1 standard deviation), this variation can be explained due to discrepancies between previously published standard values for feeds (NRC, 1996) and actual analyzed chemical composition of the major feeds used to formulate the ration (barley silage and dry-rolled barley grain). The standard values used to calculate the formulated basal diet were; 39.00% DM, 78.72% OM, 11.90% CP, 2.92% fat (ether extract), 56.80% NDF, 39.39% ADF, 14.06% starch, and 60.00% TDN for barley silage and 91.00% DM, 96.81% OM, 12.40% CP, 2.20% fat (ether extract), 20.80% NDF, 14.47% ADF, 60.70% starch, and 82.70% TDN for dry-rolled barley grain (NRC, 1996). The actual analyzed chemical composition of the barley silage contained greater amounts of OM, fat (ether extract), starch, and TDN (91.60, 3.10, 22.99, and 70.25%, respectively), lesser amounts of DM, NDF, and ADF (31.10, 47.44, and 28.14%, respectively), and similar (<1 standard deviation) amounts of CP (11.97%) (Table 13). The actual analyzed chemical composition of the dry-rolled barley grain contained greater amounts of DM and TDN (95.96 and 90.74%, respectively), lesser amounts of ADF (5.65%), and similar (<1 standard deviation) amounts of OM, CP, fat (ether extract), NDF, and starch (97.46, 13.08, 1.74, 21.23, and 59.99%, respectively) (Table 14). After further evaluation, it is clear that the variation between DM, OM, NDF, ADF, starch, and TDN in the formulated basal diet and analyzed diets is mostly due to differences in barley silage chemical composition with lesser contribution from the dry-rolled barley grain chemical composition which is directly related to the inclusion rates of these two feeds (90 vs 4.12% on a DM basis, respectively).

Analyzed chemical composition of experimental diets is presented in Table 12. Dry matter ranged from 33.97 (NOP) to 35.64% (OIL), with similar values obtained

for both CON and NOP+OIL treatments (35.31 and 35.20%, respectively) ($P = 0.002$). When compared to the non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had greater concentrations of OM (91.59 vs 91.02%, respectively) ($P = 0.0009$). These treatments also had approximately 60% greater concentration of fat (ether extract) (8.40 vs 3.16%, respectively), which was to be expected due to the inclusion of canola oil ($P < 0.0001$). Crude protein was lower for the OIL-containing treatments (OIL and NOP+OIL) (12.61 vs 13.38%) ($P < 0.0001$). Neutral detergent fiber ($P = 0.03$) and ADF ($P = 0.02$) concentrations exhibited similar patterns across treatments; both variables were greatest for NOP (43.94 and 26.10%) and different from the OIL (42.14 and 24.75%) and NOP+OIL (41.55 and 24.46%); however, all treatments were similar to the CON (43.05 and 25.33%, respectively). Starch concentration ranged from 23.76 (NOP+OIL) to 26.00% (CON), with OIL (24.55%) and NOP (25.25%) being similar to each other as well as NOP being similar to CON and OIL being similar to NOP+OIL ($P = 0.002$). The inclusion of NOP had little to no effect on analyzed chemical composition of the treatments.

There were differences between treatments for both the concentrations ($P < 0.0001$) and proportions ($P < 0.0001$) of fatty acids in the diets (Table 12). When compared to the non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had greater concentrations of saturated fatty acids (**SFA**, including C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0) (8.52 vs 5.35 mg of fatty acids/ g of sample), monounsaturated fatty acids (**MUFA**, including C16:1 *trans*-3, C16:1 *cis*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C20:1 *cis*-11, C22:1 *cis*-13, and C24:1 *cis*-15) (36.80 vs 4.16 mg of fatty acids/ g of sample),

polyunsaturated fatty acids (**PUFA**, including C18:2 n-6, C18:3 n-3, C20:4 n-6, C20:2 n-6, and C22:2 n-6) (24.86 vs 9.92), and total fatty acids (70.21 vs 19.69 mg of fatty acids/ g of sample, respectively). On a proportional basis, the OIL-containing treatments also had a greater proportion of MUFA (52.26 vs 21.19%), but lower proportions of SFA (12.09 vs 27.50%) and PUFA (35.39 vs 50.67%, respectively). The differences between the fatty acid profile of the OIL- and non-OIL-containing treatments is directly due to the inclusion of canola oil which contained 68.01 mg of SFA, 651.91 mg of MUFA, and 309.44 mg of PUFA/ g of sample or 6.06% SFA, 63.33% MUFA, and 30.07% PUFA (Table 15). The inclusion of NOP had no effect on fatty acid profile of the treatments.

The GE content was different ($P < 0.0001$) between all treatments and greatest for OIL (5.57 Mcal/kg) and NOP+OIL (5.52 Mcal/kg) and lowest for CON (5.27 Mcal/kg) and NOP (5.21 Mcal/kg) (Table 12). Although the OIL-containing treatments (OIL and NOP+OIL) had numerically greater gross energy values when compared to the non-OIL-containing treatments (CON and NOP) (5.55 vs 5.24 Mcal/kg), there was no effect of OIL. The DE content was greatest for CON (3.36 Mcal/kg) and similar between OIL and NOP+OIL (3.39 vs 3.40 Mcal/kg), however, there was no difference between NOP (3.47 Mcal/kg) and the other treatments.

The physical characteristics (particle size distribution) of the treatments are presented in Table 12. The upper (> 19 mm) and middle (8 - 19 mm) levels of the Penn State Particle Separator revealed no differences ($P > 0.05$) in particle size distribution across treatments, averaging 11.28% for the upper level and 64.68% for the middle level. However, there were differences between treatments for particles retained on the bottom level (1.18 - 8mm) in which CON (23.77%) had the most

particles retained with no differences between OIL, NOP, or NOP+OIL (22.18, 22.34, and 21.62%, respectively) ($P = 0.005$) and in the bottom pan (< 1.18 mm) in which the non-OIL-containing treatments (CON and NOP) had the greatest amount of particles when compared to the OIL-containing treatments (OIL and NOP+OIL) (2.00 vs 1.19%, respectively) ($P < 0.0001$). The particle size distribution of the barley silage was similar to the treatments in which there was 12.72, 67.31, 19.10, and 0.87% of the particles retained on the upper level, middle level, bottom level, and in the bottom pan (Table 13).

The physical characteristics of the dry-rolled barley grain are presented in Table 14. The bushel weight of the dry-rolled barley was 42.68 lbs./ bu. and had a processing index score of 81.93%. Particle size distribution was determined using the Rotap apparatus in which 14.06, 76.81, 7.91, 0.44, and 0.78% of the particles were > 4.00 mm, 2.36 - 4.00 mm, 1.18 – 2.36 mm, 850 µm – 1.18 mm, and < 850 µm in size, respectively.

The supplement mix contained 97.45% DM, 84.48% OM, 34.39% CP, 2.98% fat (ether extract), 24.78% NDF, 16.19% ADF, 11.82% starch, and 81.13% TDN (Table 16). The treatment mixes (Placebo and NOP) varied slightly in chemical composition (Table 17). When compared to the Placebo treatment mix, the NOP treatment mix contained greater concentrations of OM (86.20 vs 84.64%), lower concentrations of fat (ether extract) (16.13 vs 26.49%), NDF (23.28 vs 26.10%), and starch (37.31 vs 40.76%) and similar (< 1 standard deviation) concentrations of DM (90.90 vs 92.20%), CP (8.78 vs 9.12%), ADF (6.93 vs 6.90%), and TDN (89.56 and 89.60%, respectively).

The animal's body weights and DM, nutrient, and energy intakes are presented in Table 18. There was an effect of treatment in which animals on the OIL-containing treatments (OIL and NOP+OIL) maintained their original body weight (732.88 kg), whereas animals on the non-OIL-containing treatments (CON and NOP) lost weight during the course of the period (715.13 kg) ($P < 0.0001$). Dry matter intake was greatest for both OIL (7.35 kg/d) and NOP+OIL (7.31 kg/d), followed by CON (7.19 kg/d) and then by NOP (7.08 kg/d) ($P < 0.0001$). Nutrient intake also varied across treatments. Organic matter intake exhibited a similar pattern as to DMI in which OM intake was greatest for both OIL (6.70 kg/d) and NOP+OIL (6.66 kg/d), followed by CON (6.54 kg/d) and then by NOP (6.41 kg/d) ($P < 0.0001$). Neutral detergent fiber ($P = 0.0003$) and ADF ($P = 0.001$) were both greatest for CON (3.13 and 1.85 kg/d) and similar to OIL (3.09 and 1.82 kg/d) which was also similar to NOP (3.04 and 1.79 kg/d) which was similar to NOP+OIL (2.98 and 1.75 kg/d). Fat (ether extract) intake was different across all treatments and greatest for OIL (0.62 kg/d); followed by NOP+OIL (0.59 kg/d), CON (0.25 kg/d), and NOP (0.23 kg/d) ($P < 0.0001$). Starch intake was similar between CON (1.86 kg/d), OIL (1.83 kg/d), and NOP+OIL (1.80 kg/d), however, NOP (1.76 kg/d) was only similar to NOP+OIL ($P = 0.02$). Total digestible nutrient intake was greatest for the OIL-containing diets (OIL and NOP+OIL) (5.36 and 5.38 kg/d, respectively), followed by CON (5.21 kg/d) and NOP (5.11 kg/d) ($P < 0.0001$). Gross energy intake followed a similar pattern as to fat (ether extract) intake in which GE intake was greatest for OIL (40.78 Mcal/d); followed by NOP+OIL (40.13 Mcal/d), CON (37.90 Mcal/d), and then NOP (36.61 Mcal/d) ($P < 0.0001$). There were no treatment effects on DE intake during the course of this trial ($P = 0.27$).

The apparent total tract digestibility of the treatments was affected by treatment ($P < 0.0001$) (Table 19). When compared to non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had decreased apparent total tract digestibility of DM (60.73 vs 66.80%) and OM (61.96 vs 68.73%), with the greatest decrease in fiber digestibility; NDF (47.55 vs 60.96%) and ADF (46.47 vs 60.02%). Although OIL numerically decreased and NOP numerically increased the apparent total tract digestibility of CP, there was only a trend for significance of this parameter ($P = 0.06$). The apparent total tract digestibility of starch was not different between treatments and averaged 96.63% ($P = 0.15$).

Rumen Measurements. Diurnal pH parameters are presented in Table 20. The minimum pH was greatest for NOP (5.91), followed by CON (5.76), and then by OIL (5.66), which was similar to both CON and NOP+OIL (5.61) ($P < 0.0001$). The mean pH was greatest for NOP (6.57), followed by OIL (6.53), and similar for CON (6.49) and NOP+OIL (6.48) ($P < 0.0001$). The maximum pH was not affected ($P = 0.25$) by treatments and averaged 7.20. The pH range was greatest for OIL (1.63) and lowest for NOP (1.25), with NOP+OIL (1.57) being similar to OIL and CON (1.43) which was also similar to NOP ($P = 0.0005$). Immediately after feeding, the mean pH was highest for the OIL-containing treatments (OIL and NOP+OIL) and was then lowest around 6 h after feeding when compared to the non-OIL-containing treatments (CON and NOP) (Figure 16). In all treatments, the lowest mean pH values occurred approximately 6 h after feeding and after 12 h post-feeding, all curves behaved in a similar fashion. There were differences among treatment at all of the time points measured (0, 3, 6, 9, 12, 15, 18, and 21 h after feeding).

Ruminal *aq*H_2, ammonia-N, and VFA profile are presented in Table 21. Dissolved hydrogen was greatest for the NOP-containing treatments (NOP and NOP+OIL) when compared to the non-NOP-containing treatments (CON and OIL) (68.86 vs 9.18 µmol/L of rumen fluid) ($P < 0.0001$). The 3 and 6 h after feeding time points were the only time points in which there were differences observed among treatments (Figure 17). Dissolved hydrogen production peaked at 3 h after feeding for CON (18.86) and NOP (195.65), at 6 h after feeding for NOP+OIL (158.55), and at 9 h after feeding for OIL (23.24 µmol/L of rumen fluid). By 12 h after feeding, *aq*H_2 concentration in all treatments had decreased to approximately 0 µmol/L of rumen fluid. Ammonia-N was greatest for CON (5.21) which was similar to NOP (4.70), followed by OIL (4.33) which was also similar to NOP, and lowest for NOP+OIL (3.71 m*M*) ($P < 0.0001$). Ammonia-N concentration in the rumen fluid peaked at 3 h after feeding at a concentration of 15.12 for CON, 12.31 for NOP, 11.75 for OIL, and 9.16 m*M* for NOP+OIL and there were differences between treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 18). Total VFA concentration was greatest for CON (101.32), similar between OIL (94.78) and NOP (94.75), and lowest for NOP+OIL (88.26 m*M*) ($P < 0.0001$). There were differences in total VFA concentration between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 19). Acetate concentration was different for CON, OIL, NOP, and NOP+OIL (63.72, 55.47, 52.26, and 47.35 m*M*, respectively) ($P < 0.0001$). Acetate proportion was greatest for CON (64.27), lower for OIL (60.36), and lowest for NOP and NOP+OIL (56.93 and 55.94 mol/100 mol, respectively) ($P < 0.0001$). Both the concentration and proportion of acetate were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 20a,b). Propionate concentration was greatest for OIL (19.89) and similar to NOP

(19.45), which was also similar to NOP+OIL (18.66), which was similar to CON (18.03 m*M*) ($P = 0.001$). The proportions of propionate did not differ between OIL, NOP, and NOP+OIL (20.43, 20.49, and 20.76 mol/100 mol, respectively); however, CON was lower at 17.55 mol/100 mol ($P < 0.0001$). Both the concentration and proportion of propionate were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 21a,b). Iso-butyrate concentration was greater for CON (1.17) and lower for OIL, NOP, and NOP+OIL (1.14, 1.07, and 1.00 m*M*, respectively) ($P < 0.0001$); however, proportions of iso-butyrate were similar among treatments (1.19 mol/100 mol) ($P = 0.12$). Both the concentration and proportion of iso-butyrate were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 22a,b). Butyrate concentration was greatest for NOP (16.32), followed by NOP+OIL (14.90), and lowest for both CON and OIL (13.08 and 12.59 m*M*, respectively) ($P < 0.0001$). Proportions of butyrate were greater for the NOP-containing treatments (NOP and NOP+OIL) when compared to the non-NOP-containing treatments (CON and OIL) (15.64 vs 12.24 mol/100 mol, respectively) ($P < 0.0001$). Both the concentration and proportion of butyrate were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 23a,b). Iso-valerate was greatest for the OIL-containing treatments (OIL and NOP+OIL) (2.73 and 2.85 m*M*, respectively), lower for CON (2.32), and lowest for NOP (2.01 m*M*) ($P < 0.0001$). On a proportional basis, iso-valerate was greatest for NOP+OIL (3.18), lower for OIL (2.79), and lowest for CON and NOP (2.25 and 2.13 mol/100 mol, respectively) ($P < 0.0001$). Both the concentration and proportion of iso-valerate were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 24a,b). Concentrations of valerate were greater for the NOP-containing treatments (NOP and NOP+OIL) when compared to

the non-NOP-containing treatments (CON and OIL) (2.52 vs 2.07 m*M*, respectively) ($P < 0.0001$). Valerate proportion was different for CON, OIL, NOP, and NOP+OIL (1.80, 2.04, 2.38, and 2.58 mol/100 mol, respectively) ($P < 0.0001$). Valerate concentration was different between the treatments at 3, 6, 9, and 12 h after feeding (Figure 25a) and proportion was different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 24b). Caproate concentration was greatest for NOP (1.15) which was similar to CON (1.01), which was similar to NOP+OIL (0.94), which was similar to OIL (0.81 m*M*) ($P = 0.0007$). Caproate proportion was greatest for NOP (1.04) which was similar to NOP+OIL (0.95), which was similar to CON (0.88), which was similar to OIL (0.75 m*M*) ($P = 0.0005$). Both the concentration and proportion of caproate were different between the treatments at 3, 6, 9, and 12 h after feeding (Figure 26a,b). Overall, all VFA concentrations peaked at approximately 3 to 6 h after feeding and gradually decreased to 12 h after feeding.

The acetate : propionate ratio was greatest for CON (3.72), lower for OIL (3.09) and lowest for NOP and NOP+OIL (2.83 and 2.80, respectively) ($P < 0.0001$). The acetate + butyrate : propionate ratio was greater for CON (4.41) and lower for OIL, NOP, and NOP+OIL (3.70, 3.63, and 3.55, respectively) ($P < 0.0001$). Both the acetate : propionate and acetate + butyrate : propionate ratio were different between the treatments at 0, 3, 6, 9, and 12 h after feeding (Figure 27a,b).

There was a treatment effect for both the concentrations and proportions of fatty acids in the rumen contents 3 h after feeding (Table 22). When compared to the non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had greater concentrations of SFA (including C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0) (38.81 vs 15.64 mg of fatty acids/ g of

sample, $P < 0.0001$), *trans*-monounsaturated fatty acids (***trans*-MUFA**, including C16:1 *trans*-9, C18:1 *trans*-4, C18:1 *trans*-5, C18:1 *trans*-6, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13, C18:1 *trans*-14, C18:1 *trans*-15, and C18:1 *trans*-16) (7.63 vs 2.13 mg of fatty acids/ g of sample, $P < 0.0001$), *cis*-monounsaturated fatty acids (***cis*-MUFA**, including C16:1 *cis*-7, C16:1 *cis*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *cis*-14, C18:1 *cis*-15, C20:1 *cis*-11, C22:1 *cis*-13, and C24:1 *cis*-15) (7.86 vs 2.02 mg of fatty acids/ g of sample; $P = 0.002$), atypical dienes (including C18:2 *trans*-11 *trans*-15, C18:2 *trans*-9 *trans*-12, C18:2 *cis*-9 *trans*-12, C18:2 *cis*-9 *trans*-13, C18:2 *cis*-9 *trans*-14, C18:2 *cis*-9 *trans*-15, C18:2 *cis*-9 *trans*-16, C18:2 *trans*-9 *cis*-2, C18:2 *trans*-9 *cis*-15, C18:2 *trans*-10 *cis*-15, C18:2 *cis*-9 *cis*-15, and C18:2 *cis*-12 *trans*-15) (1.08 vs 0.33 mg of fatty acids/ g of sample; $P < 0.0001$), and total fatty acids (59.29 vs 24.66 mg of fatty acids/ g of sample; $P < 0.0001$) and lower concentrations PUFA (including C18:2 n-6, C18:3 n-3, C20:4 n-6, C20:2 n-6, and C22:2 n-6) (2.64 vs 3.21 mg of fatty acids/ g of sample; $P = 0.02$). Concentrations of branch-chain fatty acids (**BCFA**, including C15:0iso, C15:0anteiso, C16:0iso, C17:0iso, C17:0anteiso, and C18:0iso) were greatest for NOP (1.09), which was similar to CON (0.95), which was similar to both OIL and NOP+OIL (0.89 and 0.85 mg of fatty acids/ g of sample, respectively; $P = 0.01$). There were no differences between treatments for concentrations of conjugated linoleic acids (**CLA**, including C18:2 *cis*-9 *trans*-11, C18:2 *trans*-7 *cis*-9, C18:2 *trans*-10 *cis*-12, C18:2 *trans*-11 *cis*-13, C18:2 *trans*-7 *trans*-9, and C18:2 *trans*-10 *trans*-12) (0.23 mg of fatty acids/ g of sample; $P = 0.23$), conjugated linolenic acid (**CLNA**, including C18:3 *cis*-9 *trans*-11 *cis*-15) (0.06 mg of fatty acids/ g of sample; $P = 0.08$), and other fatty acids (including *cis*-11 cyclohexyl-

C17:0) (0.08 mg of fatty acids/ g of sample; $P = 0.35$). When compared to the non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had greater proportions of *cis*-MUFA (13.12 vs 8.29%; $P = 0.0006$) and lower proportions of BCFA (1.47 vs 4.13%; $P < 0.0001$), PUFA (4.42 vs 13.14%; $P < 0.0001$), and other fatty acids (0.13 vs 0.34%; $P = 0.0001$). Proportions of *trans*-MUFA were greatest for OIL (13.56), followed by NOP+OIL (12.11), and lowest for both CON and NOP (8.26 and 8.97%, respectively; $P < 0.0001$). Conjugated linoleic acid proportions were greatest for CON and NOP (0.80 and 0.76, respectively), which were similar to OIL (0.49), which was similar to NOP+OIL (0.40%; $P = 0.03$). Atypical dienes fatty acid proportion was greatest for OIL and NOP+OIL (1.77 and 1.82, respectively), and similar to CON (1.39), which was also similar to NOP (1.33%; $P = 0.02$). There were no differences between treatments for proportions of SFA (64.43%; $P = 0.31$) or CLNA (0.15%; $P = 0.33$).

Treatment affected the protozoal populations in the rumen fluid (Table 23). There was a tendency for an effect of treatment on *Isotricha* spp. in rumen fluid, which averaged 1.56×10^2 protozoa/ mL of rumen fluid ($P = 0.10$). *Dasytricha* spp. were greatest for NOP (1.33×10^3), lowest for OIL and NOP+OIL (0 and 0), and intermediate and similar for CON (8.75×10^2 protozoa/ mL of rumen fluid; $P = 0.007$). When compared to the non-OIL-containing treatments (CON and NOP), the OIL-containing treatments (OIL and NOP+OIL) had lower populations of *Entodinuim* spp. (4.43×10^4 vs 4.19×10^5; $P < 0.0001$), *Ostracodinium* spp. (0 vs 9.15×10^2; $P = 0.002$), *Metadinium* spp. (2.10×10^1 vs 7.71×10^2; $P = 0.006$), *Osphyoscolex* spp. (0 vs 2.26×10^3; $P = 0.0009$), and total protozoa (4.43×10^4 vs 4.24×10^5 protozoa/ mL of rumen fluid; $P < 0.0001$).

Rumen volume and rumen contents dry matter of animals 4 h after feeding is presented in Table 24. Rumen contents weight averaged 85.63 kg (wet basis) or 9.94 kg (dry basis) with a DM content of 11.69%. As a percentage of BW, rumen volume was 11.56% of BW (wet basis) or 1.34% of BW (dry basis). The author would like to note that although samples were collected and analyzed for DNA and RNA, these results were not included in the writing of this dissertation due to the time required for analysis in addition to the time limitations of writing this publication.

Enteric Fermentation Measurements. Methane, gH_2, and CO_2 emissions of animals fed the dietary treatments are presented in Table 25. There were differences in CH_4 emission between treatments regardless of units of expression ($P < 0.0001$). When expressed as total yield or as a proportion of BW, CH_4 emissions were 191.07 and 0.25, 140.24 and 0.20, 124.94 and 0.17, and 93.24 g/d and 0.13 g/kg of BW for CON, OIL, NOP, and NOP+OIL, respectively (Figure 28). Methane emissions peaked at approximately 3 to 6 h after feeding at 281.48 for CON, 224.23 for OIL, 179.51 for NOP, and 109.87 g for NOP+OIL (Figure 29). There were differences between treatments at all time points throughout the day. Methane, expressed as a proportion of DMI, digestible DMI (**dDMI**), and GE intake, was greatest for CON (26.24, 41.37, and 5.94), similar between OIL (19.57, 29.99, and 4.21) and NOP (17.88, 28.09, and 4.11), and lowest for NOP+OIL (12.69 g/kg of DMI, 18.81 g/kg of dDMI and 2.75% of GE intake).

Similarly, to methane emissions, gH_2 emissions were also different among treatments regardless of units of expression. Gaseous H_2 emissions were 0.00 for CON, 0.09 for OIL, 1.11 for NOP, and 0.61 g/d for NOP+OIL ($P = 0.001$) (Figure 30). Gaseous H_2 emissions peaked at 0.11 for CON, 4.17 for NOP, and 2.27 g for

NOP+OIL approximately 3 to 6 h after feeding and at 0.14 g for OIL approximately 12 to 15 h after feeding (Figure 31). There were differences between treatments at all time points throughout the day, except for 0 h after feeding. When expressed as a proportion of DMI, dDMI, GE intake, or BW, gH_2 emissions were 0.00, 0.01, 0.16, and 0.08 g/kg of DMI ($P = 0.001$); 0.01, 0.01, 0.28, and 0.20 g/kg of dDMI ($P < 0.0001$); 0.00, 0.01, 0.16, and 0.08 % of GE intake ($P = 0.0004$); and 0.00, 0.00, 0.002, and 0.001 g/kg of BW ($P < 0.0001$); for CON, OIL, NOP, and NOP+OIL, respectively.

Carbon dioxide emissions were not different when expressed as kg/d ($P = 0.30$), kg/kg of DMI ($P = 0.19$), or kg/kg of BW ($P = 0.10$). Carbon dioxide emissions peaked at approximately 3 to 6 h after feeding at 9.75 for CON, 9.55 for OIL, 9.94 for NOP, and 9.27 kg for NOP+OIL (Figure 32). There were differences between treatments at all time points throughout the day.

Urine and Feces Measurements. Volume and components of urine are presented in Table 26. Total volume was greatest for NOP (14.27) and similar for CON (11.95), OIL (12.27), and NOP+OIL (12.33 L/d; $P = 0.05$). Specific gravity was greatest for CON (1.013) and similar for OIL (1.005), NOP (1.003), and NOP+OIL (1.004; $P = 0.04$). Allantoin concentration was greatest for OIL (420.76) and NOP+OIL (397.51) and similar to CON (376.11), which was also similar to NOP (321.16 mg/L; $P = 0.008$). The OIL-containing treatments (OIL and NOP+OIL) had lower NH_3-N concentrations when compared to the non-OIL-containing treatments (CON and NOP) (3.25 vs 4.21 m*M*; $P = 0.002$). Uric acid concentration was greatest for NOP (9.51 mg/dL) and similar between CON, OIL, and NOP+OIL (8.35, 7.76, and 7.79 mg/dL, respectively; $P = 0.008$). The pH, protein, ketones, erythrocytes, urea, and nitrogen

concentrations in urine were not different between treatments and averaged 7.93 (P = 0.44), 32.03 mg/dL (P = 0.88), 3.95 mg/dL (P = 0.65), 201.83 cells/ µL (P = 0.11), 4.55 g/L (P = 0.20), and 7.21 g/L (P = 0.51).

Weight, output, chemical composition, and energy content of feces is presented in Table 27. Total weight (wet and dry) of feces was greatest for OIL and NOP+OIL, lower for CON, and lowest for NOP (13.61, 13.46, 12.30, and 10.76 kg of wet feces and 2.63, 2.64, 2.24, and 2.09 kg of dry feces (P < 0.0001), respectively). The animals on the OIL-containing treatments (OIL and NOP+OIL) had greater output of OM, NDF, and ADF (2.46, 1.81, and 1.10 kg/d (DM basis), respectively), when compared to the non-OIL-containing treatments (1.99, 1.43, 0.85 kg/d (DM basis), respectively) (P < 0.0001). Crude protein output was greatest for CON (0.32) and OIL (0.31), which was also similar to NOP+OIL (0.29), which was similar to NOP (0.28 kg/d (DM basis; P = 0.001). Starch output was lower for NOP-containing treatments (NOP and NOP+OIL) when compared to non-NOP-containing treatments (CON and OIL) (P = 0.003). Dry matter content of the feces was greatest for OIL and NOP (20.02 and 19.78), similar to NOP+OIL (19.57), and lowest for CON (19.11%, P = 0.01). Feces OM was different between all treatments and was 84.63, 86.93, 85.45, and 87.58% of DM for CON, OIL, NOP, and NOP+OIL (P < 0.0001). Crude protein content of the feces was greatest for CON and NOP (14.54 and 14.14), lower for OIL (12.10), and lowest for NOP+OIL (11.60% of DM; P < 0.0001). Fiber fractions (NDF and ADF) of the feces were also greater for animals receiving the OIL-containing treatments (OIL and NOP+OIL) (62.92 and 38.65) when compared to the non-OIL-containing treatments (CON and NOP) (59.01 and 36.44% of DM; P < 0.0001). Starch content of the feces was greatest for CON (3.04%) and NOP (2.76%), which was also similar

to OIL (2.53%), which was also similar to NOP+OIL (2.35%) ($P < 0.0001$). The animals receiving the OIL-containing treatments (OIL and NOP+OIL) also had greater amounts of gross energy in their feces as well as more gross energy output when compared to the non-OIL-containing treatments (CON and NOP) (5.58 Mcal/kg and 16.06 Mcal/d vs 5.20 Mcal/kg and 12.54 Mcal/d; $P < 0.0001$). Fecal pH was similar between CON, OIL, and NOP+OIL (7.54, 7.53, and 7.56, respectively) and lower for NOP+OIL (7.30; $P < 0.0001$).

Discussion

Methane emissions from enteric fermentation of ruminant animals, such as beef and dairy cattle, make up approximately 2.5% of the total greenhouse gas emissions in the United States and can increase global warming of the Earth (IPCC, 2013; EPA, 2015). The emission of CH_4 from animals is also a direct energy loss to the animal and can account from anywhere between 2 and 12% of GEI (Johnson and Johnson, 1995). Therefore, there is an immediate need to decrease CH_4 emissions for both environmental as well as economic reasons (Beauchemin et al., 2008).

There are a wide range of CH_4 mitigation strategies available today including genetic and management approaches to animal production; feeds, feeding management, and nutrition; and rumen fermentation modifiers. However, the magnitude of CH_4 reduction varies within these different groups. For example, rumen fermentation modifiers such as chemical inhibitors have proven to be highly effective at reducing CH_4 emissions *in vitro* and can reduce CH_4 emissions up to 100% (Knapp et al., 2014). However, they are less effective or less desirable *in vivo* due to lack of persistent inhibition effects, possible toxicity to the host animal and potentially negative effects on milk production (Liu et al., 2011; Knapp et al., 2014). Furthermore, many of the chemical inhibitors with high efficacy for CH_4 mitigation potential are not approved on a commercial level and are not generally recognized as safe (G.R.A.S.) for animals for food production and are therefore not recommended for feeding (Hristov et al., 2013). Chemical inhibitors that directly affect methanogenesis are those that are structural analogs of methyl-CoM, a unique cofactor found only in methanogenic Archaea, which is directly involved in the last step of methanogenesis (Figure 14; Lui and Whitman, 2008; Liu et al., 2011a). Structural

analogs of this molecule bind to methyl-coenzyme M reductase, the enzyme responsible for the formation of CH_4, which impedes methyl-CoM binding and thus prevents the reduction of methyl-CoM to CH_4 (Liu et al., 2011a). Until recently, a newly invented nitrooxy alkanoic derivative as a structural analog to methyl-coenzyme M, known as 3-nitrooxypropanol, was demonstrated to reduce CH_4 emissions up to 100% *in vitro* (Duval and Kindermann, 2012). Follow-up studies on the use of NOP as a CH_4 inhibitor have further been conducted *in vitro* (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2015a; Romero-Pérez et al., 2016), as well as *in vivo* in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Vyas et al., 2016a,b), dairy cattle (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015; Haisan et al., 2016; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014), with all studies exhibiting positive results for the use of NOP as a CH_4 inhibitor. The longer-term feeding studies demonstrate persistency of inhibition (Hristov et al., 2015; Romero-Pérez et al., 2015b), no toxic effects to the host animal (Romero-Pérez et al., 2015b), and no negative effect on milk production (Hristov et al., 2015).

A second method of CH_4 mitigation, the feeding of dietary fat, is one of the most extensively researched methods to lower enteric CH_4 from ruminants (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013; Jayasundara et al., 2016). The extent of CH_4 emissions due to increasing levels of fat in the diet can be variable and depends on the concentration of fat, the fatty acid composition, and the overall nutrient composition of the diets (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013). In regards to fatty acid profile of the diet, the inclusion of mono- and polyunsaturated fat have a profound effect on CH_4 production,

with little to no effect with differences in total concentration of saturated fat (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013). Unsaturated fats are toxic to fibrolytic bacteria, protozoa, and methanogens and therefore reduce their populations, that also causes a reduction in the fermentation of organic matter in the rumen (Beauchemin et al., 2008). Furthermore, unsaturated fat also serves as an alternative H_2 sink that completes with methanogens for available substrate (Johnson and Johnson, 1995). Among the various types of fat used in research to potentially lower CH_4 emissions, the use of polyunsaturated fat (oil) has been demonstrated to have the most extensive CH_4 reduction properties (Patra, 2013). Among these fats, the most common are soybean oil, linseed oil, and sunflower oil; however, canola oil has recently increased in popularity due to its economic importance and availability, specifically in western Canada, where over 18 MMT of canola are produced annually (Patra, 2013; Statistics Canada, 2016).

Therefore, the objective of this study was to determine whether a novel feed additive (NOP) and an unsaturated fat (OIL) alone and in combination could mitigate CH_4 gas emissions from cattle. The current study proved my hypothesis in which both NOP and OIL lowered CH_4 emissions from cattle, and the combined effect of NOP+OIL caused a greater effect on CH_4 reduction when compared to the individual supplementation of NOP and OIL.

The present study demonstrated total CH_4 production (g/d) and CH_4 yield (g/kg DMI or g/kg GE intake) was lowered when NOP was supplemented *in vivo* to beef cattle, supporting previous research which also demonstrated lowered CH_4 emission in beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b; Vyas et al., 2016 a,b), dairy cattle (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015;

Haisan et al., 2016; Lopes et al., 2016), and sheep (Martínez-Fernández et al., 2014). When expressed on a DMI basis, the reduction in CH_4 yield (32%) when 1.52 g of NOP/d (see "Calculations and Statistical Analysis" section for more information) was supplemented, was identical to the 32% reduction when 2.2 g of NOP/ d was supplemented observed by Hristov et al. (2015) and also similar to the 34% reduction when 1.5 g of NOP/ d was supplemented observed by Lopes et al. (2016) and the 37% reduction when 2.5 g of NOP/ d was supplemented observed by Haisan et al. (2016). The magnitude of reduction in the present study was greater than that observed in previous studies involving cattle which demonstrated a 7% reduction when 2.5 g of NOP/ d was supplemented (Reynolds et al., 2014), but also less than other studies reporting reductions of 60% when NOP was supplemented at 2.5 and 2.0 g/d, respectively (Haisan et al., 2014; Romero-Pérez et al., 2015b). The differences in CH_4 reduction between studies can most likely be explained by the differences in DMI, technique used to administer the product, basal diet characteristics, as well as the inclusion rate in the ration.

Animals (beef heifers) in the current study were fed at maintenance and averaged 7.08 kg/d of DMI for the NOP treatment, whereas Reynolds et al. (2014) reported an ad libitum DMI of approximately 19 kg/d for late lactation dairy cows. The higher DMI in the latter study, although not extremely high when compared to early or mid-lactation dairy cows, may have increased the passage rate of the liquid phase, and therefore the passage of NOP, out of the rumen. Additionally, our cows finished eating all of their TMR within the first 3 h after feeding, so although they did not consume a consistent supply of NOP throughout the day, they presumably had a slower passage rate from the rumen due to the lower intake, which would the explain

the drastic reduction in CH_4 emissions within 3 h after feeding and the residual affects present the remainder of the day.

Similar results were observed in a long-term lactation study by Hristov et al. (2015) in which lactating dairy cows had a DMI 28 kg/d on a 60:40 forage to concentrate ratio diet, but only observed a 32% decrease in CH_4 emissions when supplemented with 2.2 g of NOP/ d. Romero-Pérez et al. (2015a) supplemented beef cattle with 2.0 g of NOP/ d and observed a massive decrease in CH_4 emissions (60% reduction) when compared to the control. The animals in that experiment had a DMI of 7.14 kg/d on a 60:40 forage to concentrate ratio diet. The high DMI intake observed by Hristov et al. (2015) could have caused NOP to be washed out of the rumen as observed by Reynolds et al. (2014) with a similar amount of NOP (2.5 g of NOP/d), but not by Romero-Pérez et al. (2015b) with low intakes and similar amounts of NOP. Due to the differences in DMI intake, passage rates, and NOP inclusion rates between studies and the unknown rumen volumes, the NOP concentration within the rumen may have varied. In the current study, total rumen evacuations were conducted and rumen capacity was estimated to be approximately 76 L. We were then able to estimate the NOP concentration within the rumen to be approximately 0.17 m*M* (see "Calculations and Statistical Analysis" section for more information).

Previous studies have administered NOP using three different methods including: direct pulse dosed into the rumen at two times per day (Martinez-Fernandez et al., 2014; Reynolds et al., 2014), top-dressed onto the ration (Romero-Pérez et al., 2014), or, mixed into the ration (Haisan et al., 2014; Hristov et al., 2015; Romero-Pérez et al., 2015b; Haisan et al., 2016; Lopes et al., 2016). By mixing the product into the ration, as was done in the current study, the magnitude of CH_4 reduction

would likely be more consistent throughout the day because the animals received a continuous supply of the inhibitor, as opposed to pulse dosing the product into the rumen or topdressing the TMR (Haisan et al., 2014; Hristov et al., 2015; Romero-Pérez et al., 2015). It is also believed that pulse dosing NOP into the rumen may cause transient (not sustained) mitigation effects as demonstrated in both sheep (Martinez-Fernandez et al., 2014) and in dairy cattle (Reynolds et al., 2014) due to the fact that NOP is water soluble and is most likely washed out of the rumen with the liquid phase (Haisan et al., 2016). Although not discussed by Martinez-Fernandez et al. (2014), the possible transient effects of NOP were evident two to three h after dosing into the rumen of dairy cattle (Reynolds et al., 2014). However, Reynolds et al. (2014) demonstrated that although the drastic reduction in CH_4 emissions were only seen a few h after dosing in lactating dairy cattle, there were sustained residual effects throughout the day in which the NOP treatments had lower CH_4 emissions when compared to the control. In the current study, CH_4 emissions were highest between 0 and 6 h after feeding, and then began to drop back down to baseline levels until the next feeding, whereas Reynolds et al. (2014) demonstrated sustained CH_4 production throughout the day. Similarly, although the product was mixed into the TMR, the transient effects of NOP were most evident between 0 and 3 h after feeding, and although CH_4 emissions increased after that time, they were still relatively lower when compared to the control throughout the day. The sustained effects, or lack thereof, of NOP on CH_4 mitigation in the present study compared with Reynolds et al. (2014), which was previously described, may related back to the differences in DMI of the animals and method of administration.

The Hristov et al. (2015) study also found that when NOP was supplemented from 1.1 to 2.2 g/ d (40 to 80 mg of NOP/ kg of DM), the reduction in CH_4 only ranged from 26 to 32% and further supported by work from the same group by Lopes et al. (2016) which supplemented 1.5 g of NOP/ d (60 mg of NOP/ kg of DM) and found a 34% reduction in CH_4 emissions. Haisan et al. (2016) also observed a 23 and 37% reduction in CH_4 emissions when NOP was supplemented at 1.3 and 2.5 g/d (71 and 132 mg of NOP/ kg of DM), respectively. This data could potentially indicate that the after a certain inclusion rate, additional supplementation of NOP may not be beneficial. The study by Romero-Pérez et al (2015b) using beef cattle was similar to the current study in which both trials were conducted at the Agriculture and Agri-Food Canada Lethbridge Research and Developmental Centre (Lethbridge, Alberta Canada) under similar environmental and feeding conditions and animals consumed relatively the same amounts of DMI (7.14 and 7.08 kg/d) comprised of mainly barley silage and barley grain. Although the animals on the previous study received more NOP than those in the current study (2.0 verses 1.52 g of NOP/d), the main difference between these two studies was the forage to concentrate ratios. The previous study had a forage to concentrate ratio of 60:40 and animals were fed at ad libitum, whereas the current study had a much higher forage to concentrate ratio of 90:10 and animals were fed at maintenance based on body weight (NRC, 1996). These factors may have led to the large differences in CH_4 reduction of 60 and 32% between these two studies, respectively. Vyas et al. (2016a,b) demonstrated that animals receiving high forage diets and supplemented with 1.1 and 1.6 g of NOP/ d reduced CH_4 emissions of 23 and 29% and that animals receiving high concentrate diets and supplemented with 1.7 and 2.0 g of NOP/d reduced CH_4 emissions by 45 and 81%. It has also been

summarized by Dijkstra et al. (2017) that increases in dietary NDF content can reduce the ability of NOP to mitigate CH_4 emission, which confirmed data published by Vyas et al. (2016a,b).

Furthermore, in ruminants fed high forage diets, up to 95% of the microbial biomass is associated with feed particles (Janssen, 2010). Because solid feed particles have a slower passage rate and longer rumen residence times, as opposed to the liquid fraction, the methanogens associated with these feed particles would have slower growth rates, which would lead to a lower H_2 concentration and greater H_2 production via more thermodynamically favorable fermentation patterns to yield higher concentrations of acetate and CH_4. The high forage diet would also be conducive to yielding higher ruminal pH due to increased saliva production and increased buffering ability. An increase in ruminal pH as a result of both high forage as well as decreased passage rate would encourage decreased H_2 concentrations and subsequently increased acetate and CH_4 formation (Janssen, 2010). The optimal growing conditions for rumen methanogens are pH 6.0 to 7.5, which is at the upper range for pH of 5.6 to 6.7 in the rumen typically observed in cattle. A higher ruminal pH would facilitate methanogen growth and yield the most CH_4. A restricted level of feeding would result in a lower rate of passage, and therefore a more complete extent of digestion of fiber, which would also lead to increased acetate and CH_4 production. Additionally, although CH_4 production increases with increasing DMI, it also decreases as DMI increases above maintenance (due to increased passage rate and decrease DM digestibility), which would be unfavorable in this study (Knapp et al., 2014).

Lopes et al. (2016) suggested that the dietary forage concentration, specifically its effect on ruminal pH, may be negatively associated with the ability of NOP to

mitigate CH_4 due to the specific methanogen community present, which has been reported to be sensitive to lower ruminal pH (Hook et al., 2010). On the contrary, although the study by Romero-Perez et al. (2015b) had a lower forage to concentrate ratio, the mean ruminal pH of the animals was 6.64, whereas the mean ruminal pH in the current study was actually lower at 6.57. Therefore, although dietary forage concentration may play and important role in the efficacy of NOP, our data suggest that it may be less dependent on pH and maybe more dependent on the specific Archaeal community present due to proportionally higher forage, which would explain why our results varied from previously published data under similar conditions.

Although there are some studies that have measured the effect of NOP on total ruminal microbial populations including bacteria, methanogens, and protozoa, the results are highly variable, inconsistent, and in most cases lacking (Lopes et al., 2016). Samples of rumen contents for bacterial and methanogen populations were collected for the current study, however, at the time of writing this dissertation, the results were not yet available and were thus omitted from this publication. According to current previously published literature, total bacterial populations were not different when compared to the control in a number of studies involving sheep (Martinez-Fernandez et al., 2014), dairy cattle (Haisan et al., 2014; Haisan et al., 2016; Lopes et al., 2016), or beef cattle (Romero-Pérez et al., 2014; Romero-Pérez et al., 2015b). Similarly, among these studies and supported by results from the current study, total populations of protozoa did not differ between the NOP-supplemented animals and the control as well, except for the study by Romero-Pérez et al. (2015b) which reported an approximate 54% increase in protozoa populations which was deemed unclear by the authors and required further investigation. Total populations of methanogens were

also similar across published sources, except for Haisan et al. (2014) who reported a 65% decrease in methanogen populations with no difference in populations of bacteria or protozoa. The difference between the Haisan et al. (2014) and similar studies involving NOP was that their study contained relatively higher amounts of concentrate (38:62 forage to concentrate ratio) when compared to the 60:40 of most studies. Previous literature has confirmed that there are different microbial, specifically methanogen, populations between high and low forage diets (Hook et al., 2010; Zhou et al., 2011). It is therefore postulated that the reduction in CH_4 emissions is less correlated to total methanogen populations and more so dependent on the specific species present in the rumen (Haisan et al., 2014; Romero-Pérez et al., 2015b).

To date, there is only one study regarding the use of NOP and its effect on the specific Archaeal genus composition (Lopes et al., 2016). That study was conducted in lactating dairy cows and found no differences in total population of methanogens or proportions of *Methanobrevibacter*, *Methanosphaera*, or *Methanomicrobium* between the control animals or the animals supplemented with 1.5 g of NOP/ d even though there was a 34% reduction in CH_4 emissions between treatments. Although this data supports the fact that the methanogen population did not differ between treatments, the authors did not suggest as to why this may be, therefore more research is warranted in this area to elucidate the effects, if any, on ruminal diversity.

As previously stated, the inclusion of fat in the diet is one of the most extensively research methods to lower enteric CH_4 from ruminants (Beauchemin et al., 2008; Grainger and Beauchemin, 2011; Patra, 2013; Jayasundara et al., 2016). In reviews by Beauchemin et al. (2008), Brask et al. (2013), and Patra (2014), the relationship between the amount of added fat in the diet (% of DMI) and the reduction

in CH_4 emissions (g/kg of DMI) was observed in dairy cows, beef cattle, and sheep and it was concluded that for every 1% increase of fat in the diet, there was a 4.3 to 5.6% decrease in CH_4 emissions when expressed as a proportion of DMI. According to these guidelines, in the current study with a 5% increase in fat concentration, we would expect to see around a 22 to 28% decrease in CH_4 emissions, whereas in reality, we reported a slightly lower decrease of 25% when expressed as a proportion of DMI. Although there are many factors within the rumen to confound the effect of added fat on CH_4 emissions, the slight variation between expected and actual CH_4 reduction values was most likely due to differences in the specific fat source, the level of inclusion, the fatty acid profile, the form of the fat, and the type of diet supplied (Jenkins, 1993; Beauchemin et al., 2008). In a study in which researchers added linseed oil at 4% of DM to a 60:40 forage to concentrate ratio corn-silage based diet, they observed a 26% decrease in CH_4 production (g/kg of DMI); however, when linseed oil was added to a similar red-clover based diet, the magnitude of decrease in CH_4 production (g/kg of DMI) was only 9%, indicating that the type of the basal diet effects the efficacy of using unsaturated fat as a CH_4 mitigation strategy.

Although feeding supplemental fat in cattle diets is commonly used to increase energy density, total fat concentration within the diet should not exceed 6-7% of DMI as there is the possibility that it could depress DMI (NRC, 2001; Beauchemin et al., 2006a; Beauchemin et al., 2008). In our current study, all heifers fed the control basal diet were targeted to be fed at maintenance energy requirements according to guidelines set forth by the NRC (1996) based on the average body weight of all the animals in the experiment. Thus the OIL-containing diets were not formulated to be isoenergetic so that the inclusion of OIL to reduce CH_4 emissions is potentially

confounded with greater gross energy content of the diets due to oil. The animals fed the OIL-containing treatments consumed slightly greater amounts of DMI when compared to the non-OIL-containing treatments due to numerically greater amounts of DM within those treatments. Although the total fat content of the OIL-containing diets was around 8.40% of DM, which is above the recommended feeding rate, there did not appear to be any negative effects on intake, which was most likely due to the limited feeding of the treatments. The GE content and GE intake were also significantly different between treatments, which may indicate why the heifers receiving the OIL-containing treatments also gained more body weight when compared to heifers on the non-OIL-containing treatments. However, DE intake was similar across treatments due to the decreased digestibility of the DM, OM, NDF, and ADF fractions of the OIL-containing treatments, confirming previous data regarding supplementing unsaturated fat and its effect on digestibility (Benchaar et al., 2015). In the current study, although not measured, it was assumed that there was little to no fat in the feces and it was utilized by the animal for body weight gain, supporting the fact that the GE intakes varied, but the DE intakes were similar between treatments and heifers receiving the OIL-containing treatments were approximately 18 kg heavier than heifers receiving the non-OIL-containing treatments. Therefore, it is postulated that the supplemented oil was completely utilized by the animal as an energy source and particularly, for BW gain. Feeding high levels of unsaturated fat has also been demonstrated to decreased protein degradation in the rumen (Jenkins, 1993), which was evident in this current study in which NH_3-N concentrations were decreased in the OIL-containing treatments when compared to the CON.

Concurrent with the mode of action of feeding unsaturated fat and the decrease in digested OM within the rumen, protozoal populations were also decreased by OIL supplementation. Protozoal populations have been estimated to account for approximately 25 to 30% of total fiber digestion within the rumen (Lee et al., 2000) and are highly sensitive to the toxic effect of fatty acids (Dohme et al., 1999). A decrease in protozoa populations not only decreases the amount of fiber digested, but also decreases methanogenesis by limiting the amount of substrate available (Newbold et al., 1995). In our experiment, if we look at the total NDF intake averaged for the OIL- and non-OIL-containing treatments, account for the differences in NDF digestibility, and assume that the protozoa accounted for approximately 25% of the total fiber digestion, we can calculate the amount of NDF that was actually digested by protozoa, to find that there was a 22% decrease in the amount of NDF digested by protozoa between the OIL- and non-OIL-containing treatments (see "Calculations and Statistical Analysis" section for more information). By comparing this to the differences in total protozoa populations between the OIL- and non-OIL-containing treatments, we can see that the 18% decrease in protozoa populations may account for the majority of the 22% decrease in the amount of NDF assumed to be digest by protozoa in the rumen between the OIL- and non-OIL-containing treatments. This decrease in protozoa populations and subsequent decrease in NDF digestibility, therefore, presumably inhibited methanogenesis due to lack of available substrate, which will be discussed in detail later in the discussion.

Not only were total protozoa populations lower in the OIL-containing treatments, but specifically, the fiber digesting protozoa, including *Ostrocodinium*, *Metadinium*, and *Ophyoscolex*; were significantly reduced or almost completely

eliminated in these treatments as well. Interestingly, although *Entodinium* spp. predominantly ferments starch in the rumen (Church, 1988), they made up the majority of the total population across all treatments in the current experiment where there was only 10% (DM basis) of barley grain-based concentrate. However, these results do support previous data in an experiment conducted by Váradyová et al. (2007) in which *Entodinium* spp. were predominant in sheep which were fed a 20% (DM basis) of barley grain-based concentrate supplement and in which the population of total protozoa, *Entodinuim*, *Dasytricha*, *Isotricha*, and *Ophyoscolex* were significantly lower when linseed oil was supplemented at 5% of DM when compared to the control. This substantial reduction in ciliate protozoa is not only responsible for the large decrease in NDF digestion in the OIL-containing treatments, as discussed previously, but it also decreases the available habitat for endo- and ectosymbiotic methanogens to reside (Finlay et al., 1994). It has been estimated that these ciliate protozoa could provide a habitat for anywhere from 10 to 20% of the total rumen methanogens (Stumm et al., 1982) and these methanogens can account for up to 25% of total CH_4 produced in the rumen (Newbold et al, 1995), further indicating the ability of OIL to mitigate CH_4 emissions.

The addition of highly unsaturated fat, such as canola oil, to the diets of ruminants as a method to lower CH_4 emissions has also been associated with altering the fermentation of the rumen by providing an alternative H_2 sink (Johnson and Johnson, 1995; Beauchemin et al., 2008; Ungerfeld et al., 2015b). Unsaturated fatty acids are toxic to rumen microbes, and are therefore rapidly biohydrogenated to saturated fatty acids (Jenkins, 1993). When feed enters the rumen, the fat, mainly in the form of triglycerides (esterified fatty acids), is hydrolyzed by lipolytic

microorganisms to yield the free form of the fatty acid (NRC, 2001). These free fatty acids, being predominantly unsaturated, are first isomerized and then hydrogenated by rumen microorganisms (Jenkins, 1993; NRC, 2001). The extent of biohydrogenation depends largely on the degree of unsaturation of the fatty acids, the amount of fat in the diet, the frequency of feeding, and the specific bacterial populations within the rumen (Jenkins, 1993; NRC, 2001). In the current experiment, canola oil was supplemented to the OIL-containing treatments at 5% of DM, which was equivalent to 380 g of canola oil. Canola oil, being comprised of predominantly MUFA and PUFA (63 and 30% of total fatty acids), is highly unsaturated, and therefore, able to accept H^+ during microbial biohydrogenation. This high degree of unsaturation was apparent in the OIL-containing treatments, which not only contained approximately 51 mg of total fatty acids/ g of sample, but also had 33 and 15 mg more of MUFA and PUFA/ g of sample than the non-OIL-containing treatments, respectively. Although the non-OIL-containing treatments had proportionally more PUFA, overall these treatments had around 16 percentage units lower MUFA and PUFA (% of total fatty acids) when compared to the OIL-containing treatments. Not only did these heifers consume more unsaturated fatty acids, all the heifers in this study had completely consumed their entire TMR by approximately 3 h after feeding. Therefore, the majority of unsaturated fatty acids entered the rumen within a relatively short time frame. This rapid increase in substrate and unsaturated fatty acids in the rumen may also explain why CH_4 emissions peaked at 3 to 6 h after feeding and then began to decline after that time in the heifers receiving the OIL treatment. Due to the time and cost limitations of analysis, only the 3 h sampling time point was selected for the fatty acid profile determination of the rumen contents, it is therefore unknown as to what time point

biohydrogenation peaked in relation to CH_4 production. Regardless, this study demonstrated that after 3 h post-feeding, the rumen contents of the heifers receiving the OIL-containing treatments were similar to the original TMR in which there were proportionally more MUFA (*cis* and *trans*) and less PUFA. However, there were similar proportions of SFA in the rumen contents of heifers receiving the OIL- and non-OIL-containing treatments, whereas the original OIL-containing treatments contained proportionally less SFA. These data indicate that the greater amounts overall of MUFA and PUFA supplied by the OIL-containing treatments were biohydrogenated to SFA and consequently were able to use more H_2 from the rumen environment at this time point.

Although the specific microbiome of the bacterial population of the rumen of heifers used in this study is unknown, it is evident that the biohydrogenation of unsaturated fatty acids served as an alternative H_2 sink by absorbing excess H_2 within the rumen; however, this process has only been estimated to account for 1 to 2% of H_2 absorption (Czerkawski and Clapperton, 1984; Jenkins, 1993). These results demonstrate the collective properties of supplementing an unsaturated fat, such as canola oil, and its ability to reduce the amount of organic matter fermented, reduce protozoal populations, and serve as a minor alternative H_2 sink; which thereby decreased CH_4 emissions by 25% when expressed as a proportion of DMI.

This study supports previous research regarding the individual supplementation of a novel CH_4 inhibitor, NOP, and a known CH_4 mitigant, canola oil, as a means to lower CH_4 emissions in ruminants. However, this study was novel in the fact that it was the first of its kind to demonstrate the effectiveness of NOP (a chemical inhibitor) in combination with canola oil (a feed supplement) on CH_4

emissions *in vivo* in beef cattle. To date, the only similar study that was conducted was by the same group of researchers, who observed the individual and combined effects of NOP and monensin (**MON**; a feed additive) and their ability to lower CH_4 emissions *in vitro* due to different modes of action (Romero-Pérez et al., 2016). In that experiment, both NOP and MON were supplemented individually (NOP, MON) and combined (NOP+MON) at 2 mg/d in a 60:40 forage to concentrate ratio, barley-based ration in RUSITEC fermenters. On the final day of the treatment period, CH_4 emissions (mL/d) were decreased by 72, 12, and 70% for the NOP, MON, and NOP+MON treatments when compared to the control. Although both NOP and MON were efficacious at reducing CH_4 emissions, with the latter being less effective, contrary to their hypothesis, the two supplements did not exhibit an additive effect when combined, but, there was a 66 and 83% increase in gH_2 emissions (mL/d) for the NOP and NOP+MON treatments, respectively. However, the increase in gH_2 emissions in the NOP treatment only accounted for 15% of the H_2 not incorporated into methane, indicating other sinks of hydrogen in the rumen compensated additional hydrogen available due to the reduction in CH_4 emissions. A meta-analysis by Ungerfeld (2015a) suggested that in addition to the increase in gH_2 emissions and lack of difference in propionate production (data not shown), alternative H_2 sinks within the fermenters can utilize excess H_2, including formate, succinate, ethanol, microbial biomass, and reductive acetogenesis.

Although both monensin and canola oil reduce the amount of H_2 produced in the rumen, they have different modes of action. Monensin is commonly used in beef and dairy cattle diets to increase feed efficiency by decreasing populations of H_2-producing bacteria and shift fermentation to more energy efficient pathways, such as

propionate production; however, its theoretical ability to lower CH_4 emissions is negligible (Russel and Houlihan, 2003). On the contrary, canola oil has the ability to lower CH_4 emissions, by the previously discussed modes of action, and when supplemented in combination with NOP, as in this current study, has been demonstrated to lower CH_4 emissions by 52% when presented as a g of CH_4/ kg of DMI basis. Unlike the study by Romero-Pérez et al. (2016), the combined effects of both NOP and OIL were more effective in lowering CH_4 emissions then the individual supplementation of both these additives. However, the combination of NOP and OIL did not prove to have an additive effect for lowering CH_4 emissions, in which we would expect to see a 57% decrease in CH_4 emissions. Therefore, it is presumed that the substantial reduction in CH_4 emissions in the NOP+OIL treatment was caused by NOP blocking the active site of the methyl-coenzyme M reductase enzyme in addition to the OIL reducing the amount of OM fermented, reducing protozoa populations, and serving as an alternative H_2 sink which reduced overall substrate (H_2) availability which would otherwise be used for methanogenesis.

In the rumen, methanogenesis not only acts as the primary H_2 sink by absorbing H_2, but it also re-oxidizes co-factors (NAD^+) involved in glycolysis, allowing fermentation to continue (Hungate, 1967; Ungerfeld, 2015a). The H^+ produced as a byproduct of fermentation is acted upon by hydrogenase-expressing bacteria and converted to H_2 within the rumen (Knapp et al., 2014). Carbon dioxide, in combination with H_2, are the main substrates for CH_4 production in the rumen. Carbon dioxide emissions (kg/kg of DMI) were similar across treatments, indicating that CO_2 was not lost from the rumen and was most likely used as substrate and incorporated into alternative pathways in the rumen without accumulating, confirming

previous similarities in other experiments (Hristov et al., 2015; Lopes et al., 2016). The heifers in the current experiment emitted approximately 8.21 kg of CO_2/d, which was slightly higher than previously reported values of 7.34 kg/d (Romero-Pérez et al., 2014). However, in the previous study, the animals were smaller in size (549 kg) and consumed approximately 8.50 kg of DMI/d where as in the current study; animals averaged 732 kg in body weight and consumed 7.08 to 7.35 kg of DMI/d. The larger mass of these animals would coincide with an increased metabolic rate, and therefore, these animals would be expected to produce more CO_2 emissions (McGinn et al., 2004).

Concurrent with the reduction in CH_4 emissions in the present experiment, H_2 gas emissions were increased in treatments supplemented with NOP. When compared to the CON, H_2 emissions (g/kg of DMI) were greatest for the NOP treatment and intermediate for the NOP+OIL treatment, with the OIL treatment being similar to CON. Although few studies have been conducted using NOP where emission of H_2 has been measured, a couple studies reported increased emissions when compared to the control (Hristov et al., 2015; Lopes et al., 2016). In the study by Hristov and others (2015), researchers reported *g*H_2 yields in lactating dairy cattle of 0.00, 0.48, 0.96, and 1.27 g/d without NOP supplementation and when NOP was supplemented at 1.1, 1.7, and 2.2 g/d, respectively. In a follow up study, similar *g*H_2 yields of 1.3 g/d were reported by Lopes et al. (2016) when NOP was supplemented at 1.5 g/d. Both the highest inclusion rate of NOP in the study by Hristov et al. (2015) and in the study by Lopes et al. (2016) reported similar values of *g*H_2 yields (1.27 and 1.30 g/d) as well as similar magnitudes of CH_4 reduction (-32 and -34%) when compared to the control and expressed on a DMI basis. These previous results were also similar to our

experiment in which NOP was supplemented at 1.52 g/d, induced a -32% decrease in CH_4 emissions when compared to the control, and increased *g*H_2 yield by 1.11 g/d. Although these studies were similar in magnitude of CH_4 reduction and *g*H_2 yield, the amount of *g*H_2 eructated by these animals did not account for the total H_2 which was spared from CH_4 production. For example, both Hristov et al. (2015) and Lopes et al. (2016) reported that the *g*H_2 that was eructated by the animal only accounted for approximately 2-3% of the H_2 spared from CH_4 production. In our study, the *g*H_2 eructated by the heifers accounted for 1, 7, and 2% of the H_2 spared from CH_4 production for the OIL, NOP, and NOP+OIL treatments (see "Calculations and Statistical Analysis" section for more information). The large differences between the proportions of H_2 accounted for between the treatments, with the OIL-containing treatments being substantially lower than the non-OIL-containing treatments, is further indicative of the ability of unsaturated dietary fat to act as a hydrogen sink in the rumen. Interestingly, and unknown as to why, the proportion of *g*H_2 recovered in our heifers that received the NOP treatment alone was more than double that previously reported by Hristov et al. (2015) and Lopes et al. (2016). This raises questions as to why similar reductions of CH_4 and increases of *g*H_2 yields occurred, yet the recoveries of *g*H_2 varied among studies. There were a few slight differences between the current experiment and the experiments by Hristov et al. (2015) and Lopes et al. (2016) that also used NOP. These differences may have led to differences in availability of alternative H_2 sinks within the rumen, and this the emission of *g*H_2.

Previous research has demonstrated that the specific proportions of VFA produced and the formation of CH_4 are related to the ability of the rumen environment to absorb and utilize H_2 and the partial pressure of H_2 (concentration of dissolved H_2)

in the rumen (Czerkawski et al., 1972; Hegarty and Gerdes, 1999; Janssen, 2010). The H_2 balance within the rumen is directly responsible for ruminal fermentation via dynamic control of growth kinetics, thermodynamic control of fermentation pathways, and stoichiometric control of end-production formation (Hungate, 1967; Janssen, 2010). Although all pools of H_2 influence ruminal fermentation, only *aq*H_2 is biologically available for utilization into H_2 sinks such as methanogenesis (Janssen, 2010). Currently, limited data are available, the only accurate way to estimate *aq*H_2 is to measure it directly from the rumen fluid; which has been summarized to range from 0.1 to 50 μ*M* in non- CH_4 inhibited rumens (Janssen, 2010). In the current study, *aq*H_2 concentration averaged 7.32, 11.02, 71.60, and 66.12 μ*M* for the CON, OIL, NOP, and NOP+OIL treatments, respectively. The *aq*H_2 concentration for the CON treatment is within the range of non-CH_4 inhibited rumens from data summarized by Janssen (2010) and similar to our eructated *g*H_2 production (g/d) data, in which the NOP-containing treatments were greater than the non-NOP-containing treatments. Interestingly, differences in both *aq*H_2 and *g*H_2 concentrations were relative between treatments, but they also peaked at 3 h post feeding for the NOP treatment and 3 to 6 h postfeeding for the NOP+OIL treatment, with the CON and OIL treatments being low and relatively similar thoughout the sampling periods. Therefore, although the *g*H_2 concentration of the headspace (which is assumed to be eructated) cannot be used to estimate the *aq*H_2 concentration of the rumen fluid (Wang et al., 2016b), it could have the potential to estimate it when *aq*H_2 concentration is highest in the rumen, and vice versa.

To date, there is only one published study (Lopes et al., 2016) with supplemented NOP *in vivo* that has attempted to measure *aq*H_2 concentration in the

rumen fluid. However, researchers found no difference ($P = 0.27$) in aqH_2 concentration between the control cows and those supplemented with 1.5 g of NOP/d (0.02 and 1.2 mg/L, which is equivalent to approximately 99 and 594 μ*M*). Their samples were collected approximately 3 to 4 h post-feeding and were greater in concentration, but with smaller differences in magnitude (approximately a 6-fold difference) when compared to the aqH_2 concentration of CON and NOP in the current study, which were 19 and 196 μ*M* (approximately a 10-fold difference) at the 3 h post-feeding time point. The overall greater values observed by Lopes and colleagues (2016) were most likely due to differences in rumen dynamics; such as DMI, passage rate, and abundance of available substrate, between lactating dairy cattle and the beef heifers in the current study.

In the study by Lopes and others (2016), although there was a numerical increase in aqH_2 concentration in the rumen fluid of cows supplemented with NOP, which would be expected with the significantly higher eructated gH_2 yields, the differences between treatments were not significant due to "extremely variable" data. The inconsistent data observed with this parameter were mostly likely due to the small sample size (n = 6 cows). Furthermore, and more likely, the variability and lack of differences between treatments could be explained by the potential confounding effects from the sampling, handling, and analysis technique used (Lopes et al., 2016; VaporTech Environmental Laboratory Services, 2017). Therefore, based on the referenced literature, the aqH_2 concentrations reported by Lopes et al. (2016) should be interpreted with caution.

The H_2 balance within the rumen can influence the thermodynamic control of fermentation pathways, which was briefly discussed earlier (Hungate, 1967; Janssen,

2010). For example, ruminal microorganisms have the ability to change the pathways that they utilize to ferment feeds depending on the concentration of H_2 in the rumen (Janssen, 2010). When the concentration of H_2 is high, the utilization of pathways that produce H_2 become thermodynamically unfavorable, and therefore, the amount of H_2 produced decreases (Janssen, 2010). However, when the concentration of H_2 is low, these H_2-producing pathways become more thermodynamically favorable, and the amount of H_2 produced increases (Janssen, 2010). Although the fermentation of glucose to VFA is dependent on the concentration of the substrate, not all pathways are equally effected by the change in H_2 concentration. This was demonstrated by the change in Gibbs free energy between reactants and products (ΔG_T) over a range of substrate (H_2) concentrations (Janssen, 2010). ΔG_T is more negative, with more energy being released and available for growth of microorganisms, at low concentrations of H_2 compared to higher concentrations of H_2, therefore H_2-producing pathways are more energetically favorable (acetate) (Janssen, 2010). On the contrary, at high concentrations of H_2, ΔG_T is more positive, therefore, H_2-utilizing pathways are more energetically favorable (propionate and butyrate) (Janssen, 2010).

In the current study, heifers receiving the NOP-containing treatments (NOP and NOP+OIL) had decreased proportions of acetate (-11 and -13%) and increased proportions of propionate (+17 and +18%) and butyrate (+31 and +27%, respectively) when compared to the CON treatment. Consequently, these treatments were also associated with lower acetate:propionate ratios (-24 and -25%, respectively). The results presented here confirm previous studies by Janssen (2010), McAllister and Newbold (2008), and Ungerfeld (2015b) where methanogenesis was inhibited and also supports previous studies that supplemented NOP (Martinez-Fernandez et al., 2014;

Reynolds et al., 2014; Romero-Pérez et al., 2015b; Lopes et al., 2016; Haisan et al., 2016). For example, in studies with similar magnitudes of CH_4 reduction, when 1.5 g of NOP/ d was supplemented, researchers reported a decrease in acetate proportion of -6% and an increase in proportions of propionate by +5% and butyrate by +18% (Lopes et al., 2016). They also reported a decrease in the acetate:propionate ratio by –11%. In a comparable study by Haisan et al. (2016), authors reported a -10, +11, and +15% differences in proportions of acetate, propionate, and butyrate, respectively, when 2.5 g of NOP/d was supplemented. Those authors also reported a 19% decrease in the acetate:propionate ratio. Interestingly, among all those of these studies, butyrate proportions exhibited the greatest magnitudes of increase when compared to the magnitudes of change in the acetate and propionate ratios. However, overall, heifers receiving the NOP treatment in the current study demonstrated the greatest decrease in acetate and increase in propionate and butyrate proportions when compared to the CON treatment. These animals also had 6% lower concentrations of total VFA when compared to animals on the CON treatment, which usually indicates decreased digestion; however, apparent total tract digestibilities were similar between the CON and NOP treatments. In similar studies conducted by Romero-Pérez et al. (2014; 2015b) a numerical reduction in total VFA concentration was reported, with either no difference in digestibility or no measurement of digestibility, respectively. Reynolds et al. (2014) reported a decrease in total VFA concentration, and only had a tendency for reduced DM, OM, CP, and ADF digestibility. On the contrary, Hristov et al. (2015) found no difference in diet digestibility, but did not measure rumen parameters and Haisan et al. (2016) reported similar or increased DM and NDF digestibilities, but no differences in VFA concentration. Therefore, although limited studies reported no

differences or decreases in total VFA concentration within the rumen, due to lack of data regarding diet digestibility parameters, it is difficult to find conclusive evidence as to say whether the inclusion of NOP negatively effects ruminal fermentation. However, it has been suggested that the decrease in VFA concentration with no effect on total tract digestion may be due to increased post-ruminal digestion and/ or increase absorption/ passage rate from the rumen (Romero-Pérez et al., 2015b). It is well known that high levels of unsaturated fat can decrease diet digestibility (Beauchemin and McGinn, 2006b), which was apparent in this study and supports the fact that there were lower total VFA concentrations in the OIL-containing treatments which were lowest for the NOP+OIL treatment and most likely due to the combined effect of the additives.

Minimum pH values were greater for the NOP treatment when compared to the CON treatment. Previous studies by Reynolds et al. (2014) and Romero-Pérez et al. (2014; 2015b) have reported increases in minimum pH when compared to the control and it has been suggested that this increase was due to a decrease in total VFA concentration, which would coincide with our results for the NOP treatment. Interestingly, and unknown as to why, the current study was also the first to report an increase in the mean pH between the NOP and CON treatments. However, another study by Haisan et al. (2016) reported no difference in minimum, mean, or maximum pH, indicating that more data needs to be generated prior to forming a conclusion regarding the effects of NOP, if any, on ruminal pH. One explanation as to why the mean ruminal pH was greater in the NOP treatment could be due to the microbial population of the rumen, specifically the methanogens, and the methanogenesis pathways that these organisms use. For example, Duin et al. (2016) found that NOP

does not inhibit all methanogens equally, with *Methanosarcine barkeri* and *Methanomicrobium mobile*, requiring a one-hundred times greater concentration of NOP than *Methanobrevibacter ruminatium* to inhibit methanogenesis *in silico* and *in vitro*. Although we don't know the specific methanogen populations in the current study, if we assume that the majority of the population belong to the *Methanobrevibacter* genera (Janssen and Kirs, 2008) which employs the hydrogenotrophic pathway and uses CO_2 and H_2 and some formate as substrates to produce CH_4, inhibition of this pathway could cause an increase in available substrate. According to our data, eructated *g*H_2 was increased in the NOP treatment, but there were no differences in eructated CO_2 yield between treatments, therefore postulating the belief that this carbon source was utilized within the rumen. In theory, the CO_2 that wasn't used for CH_4 production could have combined with water to form bicarbonate, which may have acted as a buffer in the rumen which would support the increase in minimum and mean pH and *aq*H_2 concentration in the NOP treatment (Romero-Pérez et al., 2015a). This would also explain why methanogenesis was only inhibited and not completely eliminated and why our results differ from previously published pH data. However, it is still unclear as to why the NOP+OIL treatment had the lowest mean pH and the least total VFA, when compared to the CON treatment, which had a similar pH, but the greatest VFA concentration.

Methane production is one of the major H_2 sinks in the rumen, however, when methanogenesis is inhibited, there are alternative pathways that can utilize and prevent accumulation of H_2. Although this current study was not designed to determine H_2 production, we can postulate the possible alternative pathways in which this H_2 could have been utilized. These alternative H_2 sinks include another major H_2 sink, fumarate

reduction, and minor H_2 sinks including biohydrogenation of unsaturated fatty acids, formate production, reductive acetogenesis, nitrate and nitrite reduction, and sulfate reduction.

The second largest H_2 sink in the rumen is the reduction of fumarate via fumarate-reducing bacteria (Kobayashi, 2010; Ungerfeld et al., 2014). These bacteria, including *Fibrobacter succinogenes, Selemonas ruminantium, Veillonella parvula* and *Wolinella succinogenes,* can compete directly with methanogens for substrates, including H_2 and formate (Asanuma et al., 1999b; Castillo et al., 2004). Fumarate is first reduced to succinate and then succinate is converted to propionate. The formation of propionate within the rumen is a viable alternative use of H_2 in which the end-product can be utilized by the animal for energy (Castillo et al., 2004; Ellis et al., 2008). According to our results, CH_4 production was decreased and propionate proportions were increased in the OIL, NOP, and NOP+OIL treatments, indicating that the reduction of fumarate was one of the alternative H_2 pathways in the current experiment.

As previously discussed, the biohydrogenation of unsaturated fatty acids can also serve as a minor H_2 sink and can account for 1 to 2% of H_2 absorption (Czerkawski and Clapperton, 1984; Jenkins, 1993). Although it is unknown as to the percentage of H_2 absorbed from this process, the OIL-containing treatments had similar amounts of saturated fatty acids as the non-OIL-containing treatments, indicating that biohydrogenation did serve as an alternative H_2 sink when canola oil was supplemented.

Although not measured in the current study, excess H_2 may have been absorbed in the production of formate (Stewart et al., 1997; Ungerfeld, 2015b). Under

normal conditions, formate is metabolized in the production of CH_4 (Hungate et al., 1970), however, it has been demonstrated to accumulate when methanogenesis is inhibited (Asanuma et al., 199a,b; Ungerfeld, 2015a). The production of formate not only utilized H_2, but CO_2 as well, which may also explain the lack of differences in eructated CO_2 between treatments.

Acetogenic bacteria within the rumen can theoretically compete with rumen methanogens for H_2 as an energy source. Although the formation of acetate within the rumen appears to a viable alternative use of H_2 in which the end-product can be utilized by the animal for energy, this pathway is not commonly employed within the rumen (Ungerfeld, 2015a). Theoretically, if CH_4 production was completely inhibited and reductive acetogenesis was to occur, we would expect to see an increase in acetate production, however, in the current study, CH_4 production was lowered, and the concentration of aqH_2 was increased in the NOP-containing treatments, but, there was also a decrease in acetate proportion. Our results confirm previous data in a recent meta-analysis regarding shifts in the H_2 sinks in methanogenesis-inhibited ruminal fermentation in batch and continuous-cultures. Ungerfeld (2015a) proposed that one of the H_2 sinks that was absorbing the excess H_2 was acetate via reductive acetogenesis; however, this researcher did not conclude an increase in acetate concentration, therefore suggesting that the H_2 was utilized in another H_2 sink.

The reduction of SO_4^{-2} is also considered a minor H_2 sink within the rumen (Morgavi et al., 2010) due to low SO_4^{-2} concentrations in normal functioning rumens. Unless SO_4^{-2} is supplemented in the ration, very little SO_4^{-2} is present within the rumen unless it is consumed within the ration itself. Regardless, the diets in the current study were comprised of 90% barley silage and 5% dry-rolled barley grain,

both of which contain approximately 1.16 and 1.70% methionine (CP basis), and 0.66 and 2.28% cysteine (CP basis), which only equates to about 0.17 and 0.12% sulfur (DM basis) (NRC, 2001), respectively; therefore, sulfate reduction as an alternative H_2 sink is unlikely.

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Chapter 3

OVERALL CONCLUSIONS

The overall objective of this study was to determine that a novel feed additive (3-nitrooxypropanol) and an unsaturated fat (canola oil) alone and in combination can mitigate CH_4 gas emissions from cattle. We hypothesized that both 3-nitrooxypropanol and canola oil would lower CH_4 emissions from cattle, and the combined effect of 3-nitrooxypropanol + canola oil will cause a greater effect on CH_4 reduction when compared to the individual supplementation of 3-nitrooxypropanol and canola oil. We confirmed our hypothesis and the results on the individual inclusion of a novel feed additive, 3-nitrooxypropanol, and an unsaturated fat, canola oil, supports previous data regarding the efficacy of these two supplements to mitigate CH_4 emissions from cattle. This study was novel in the fact that it is the first of its kind to combine 3-nitrooxypropanol and a second well known CH_4 mitigant, canola oil, and to demonstrate their combined ability to lower CH_4 emissions in vivo in beef cattle. Although there has been one study conducted in vitro which observed the individual and combined effects of 3-nitrooxypropanol and monensin on CH_4 mitigation, the combination of the two additives did not produce a greater effect as compared to the individual ingredient supplementation. Furthermore, only 1, 7, and 2% of the spared H_2 was accounted for as gH_2 in the OIL, NOP, and NOP+OIL treatments, indicating the role of alternative H_2 sinks in the rumen when methanogenesis is inhibited. Methanogenesis inhibition by NOP resulted in the synthesis of other H_2-utilizing compounds, and due to the lack of H_2 sinks, there was a small increase in gH_2 and aqH_2 accumulated in the rumen. However, the increase in aqH_2 concentration did not appear to affect digestibility of the treatments. The

inclusion of 3-nitrooxypropanol in combination with canola oil, lowered CH_4 emissions to an even greater extent due to decreased protozoa populations and diet digestibility resulting in less overall available substrate and by acting as a minor H_2 sink via biohydrogenation of unsaturated fatty acids. Although this study adds more data and understanding of the knowledge base surrounding 3-nitrooxypropanol, more research is warranted to determine its effects on the specific methanogen populations within the rumen and interactions with other feed ingredients or CH_4 mitigation strategies.

TABLES

Table 1 U.S. N_2O and CH_4 emissions from agriculture in 2013.

Source	MMT CO_2 Eq.	% of total
N_2O	**281.10**	**54.52**
Agricultural Soil Management	263.70	51.14
Manure Management	17.30	3.36
Field Burning of Agricultural Residues	0.10	0.02
CH_4	**234.50**	**45.48**
Enteric Fermentation	164.50	31.90
Manure Management	61.40	11.91
Rice Cultivation	8.30	1.61
Field Burning of Agricultural Residues	0.30	0.06

(IPCC, 2013; EPA, 2015).

Table 2 Known species of methanogenic Archaea.

Order	Family	Genus	Species	Substrate Utilization
Methanobacteriales	Methanobacteriaceae	*Methanobrevibacter*	*M. smithii*	H_2, formate
			M. ruminantium	H_2, formate
			M. thaueri	H_2, formate
		Methanobacterium	*M. aarhusense*	H_2, formate
			M. formicicum	H_2, formate
		Methanosphaera	*M. stadtmanae*	H_2, methanol
Methanomicrobiales	Methanomicrobiaceae	*Methanomicrobium*	*M. mobile*	H_2, formate
Methanomassiliicoccales	Methanomassiliicoccaceae	*Methanoplasmatales*	Unknown	H_2, methanol, methylamine
Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	*M. barkeri*	H_2, methylamine, acetate

(Liu and Whitman, 2008; Kong et al., 2013; Lang et al., 2015; Huang et al., 2016).

Table 3 Methanogenesis reactions.

Reaction	ΔG′° (kJ/mol)
Carbon dioxide	
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135
$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-130
CO_2 + 4isopropanol → CH_4 + 4acetone + $2H_2O$	-37
$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	-196
Methylated C1 compounds	
$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-105
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
$2(CH_3)_2\text{-}S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S$	-49
$4CH_3\text{-}NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$	-75
$2(CH_3)_2\text{-}NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$	-73
$4(CH_3)_3\text{-}N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$	-74
$4CH_3NH_3Cl + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH4Cl$	-74
Acetate	
$CH_3COOH \rightarrow CH_4 + CO_2$	-33

(Zinder, 1993; Hedderich and Whitman, 2006; Liu and Whitman, 2008).

Table 4 Summary of the effects of 3-nitrooxypropanol as a methane inhibitor (studies from 2014 to 2015).

	Study[1]					
Item	1	2	3	4	5	6
Location of study	Spain	Canada	United Kingdom	Canada	United States	Canada
Species	Sheep	Dairy cattle	Dairy cattle	Beef cattle	Dairy cattle	Beef cattle
% of CH_4 (g/kg of DMI) change	-24	-60	-4, -7	ns, ns, -33	-26, -31, -32	-60
NOP dose						
g/d	0.1	2.5	0.5, 2.5	0.5, 1.4, 2.8	1.1, 1.7, 2.2	2.0
mg/kg of DM	111	130	25, 125	57, 163, 346	40, 60, 80	280
Supplementation method	2x/d pulse dose	mixed into TMR	2x/d pulse dose	top-dressed onto TMR	mixed into TMR	mixed into TMR
Forage: concentrate	60:40	38:62	51:49	60:40	60:40	60:40
DMI, kg/d	0.9	19.3	18.8, 18.5	8.7, 8.6, 8.1	28, 27.7, 27.5	7.14
% of DMI (kg/d) change	ns	ns	ns	ns, -6, ns	ns	ns

(modified from Beauchemin (2014)).

[1]Studies are as follows: 1= Martinez-Fernandez et al. (2014), 2= Haisan et al. (2014), 3= Reynolds et al. (2014), 4= Romero-Perez et al. (2014), 5= Hristov et al (2015). 6=Romero-Perez et al. (2015b).

ns= not significantly different from the control treatment.

Table 5 Summary of the effects of 3-nitrooxypropanol as a methane inhibitor (studies from 2016 to present).

	Study[1]			
Item	7	8	9	10
Location of study	Canada	United States	Canada	Canada
Species	Dairy cattle	Dairy cattle	Beef cattle	Beef cattle
% of CH_4 (g/kg of DMI) change	-23, -37	-34	backgrounding: ns, -29; finishing: -9, -81	High forage: ns, ns, -16, -21, -23; High grain: ns, ns, -26, -33, -45
NOP dose				
g/d	1.3, 2.5	1.5	backgrounding: 0.9, 1.6; finishing: 1.0, 2.0	High forage: 0.33, 0.49, 0.67, 1.0, 1.1; High grain: 0.38, 0.61, 0.84, 1.1, 1.7
mg/kg of DM	71, 132	60	100, 200	50, 75, 100, 150, 200
Supplementation method	mixed into TMR	mixed into TMR	mixed into TMR	mixed into TMR
Forage: concentrate	60:40	56:44	backgrounding: 70:30; finishing: 8:92	High forage: 65:35; High grain: 8:92
DMI, kg/d	18.3, 18.9	24.8	backgrounding: 8.76, 8.15; finishing: 10.21, 9.86	High forage: 6.54, 6.47, 6.73, 6.73, 5.53; High grain: 7.64, 8.08, 8.37, 7.50, 8.49

Summary of the effects of 3-nitrooxypropanol as a methane inhibitor (studies from 2016 to present) (continued).

	Study[1]			
Item	7	8	9	10
% of DMI (kg/d) change	ns, ns	ns	backgrounding: ns, -7; finishing: ns, ns	High forage: ns, ns, ns, ns, ns; High grain: ns, ns, ns, ns, ns;

(modified from Beauchemin (2014)).
[1]Studies are as follows: 7= Haisan et al. (2016), 8= Lopes et al. (2016), 9= Vyas et al. (2016a), 10= Vyas et al. (2016b).
ns= not significantly different from the control treatment.

Table 6 Animal treatment assignments.

Group	Pair	Animal Number	Body Weight[1], kg	Period	Treatment[2]
1	1	21	701	1	CON
				2	OIL
				3	NOP+OIL
				4	NOP
	2	6	743	1	OIL
				2	NOP
				3	CON
				4	NOP+OIL
	3	2	789	1	NOP
				2	NOP+OIL
				3	OIL
				4	CON
	4	9	718	1	NOP+OIL
				2	CON
				3	NOP
				4	OIL
2	1	11	739	1	CON
				2	OIL
				3	NOP+OIL
				4	NOP
	2	12	754	1	OIL
				2	NOP
				3	CON
				4	NOP+OIL
	3	8	641	1	NOP
				2	NOP+OIL
				3	OIL
				4	CON
	4	10	770	1	NOP+OIL
				2	CON
				3	NOP
				4	OIL

Animal treatment assignments (continued).

[1]Body weights were based on measurements taken at the beginning of the Training Period.
[2]CON= control treatment, OIL= canola oil treatment, NOP= 3-nitrooxypropanol treatment, NOP+OIL= 3-nitrooxypropanol and canola oil treatment.

Table 7 Summary of experimental timeline for Group 1 and Group 2.

Group	Period	Start Date	End Date
1	Training	January 20, 2016	February 4, 1016
	1	February 5, 2016	March 3, 2016
	2	March 4, 2016	March 31, 2016
	3	April 1, 2016	April 28, 2016
	4	April 29, 2016	May 27, 2016
2	Training	January 27, 2016	February 11, 1016
	1	February 12, 2016	March 10, 2016
	2	March 11, 2016	April 7, 2016
	3	April 8, 2016	May 5, 2016
	4	May 6, 2016	June 3, 2016

Table 8 Ingredient composition (% of DM) of basal diet.

Ingredient[1]	% of DM
Barley Silage	90.000
Barley grain, dry rolled	4.120
Treatment mix[2]	0.880
Canola meal	3.300
Urea[3]	0.100
Limestone[4]	0.300
Vitamin and mineral premix[5]	0.050
Molasses[6]	0.500
Barley grain, ground	0.744
Vitamin E (500,000 IU/kg)[7]	0.006

[1]All ingredients except barley silage, dry rolled barley grain, and the treatment mix were included in a supplement mix. Forage and grains were locally sourced from the Southern Alberta region. Vitamins and minerals were purchased from a local supplier (ADM Alliance Nutrition, Lethbridge, AB Canada).
[2]Treatment mixes contained 63.19% ground barley, 14.20% canola oil (Richardson International, Winnipeg, MB Canada), and 22.61% of either 8.85% 3-nitrooxypropanol on silicon dioxide and propylene glycol (NOP and NOP+OIL treatments) or a placebo mix containing silicon dioxide and propylene glycol (Training Period basal diet, CON, and OIL treatments) (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland).
[3]Pestell Minerals and Ingredients, New Hamburg, ON Canada.
[4]Graymont Ltd., Calgary, AB Canada.
[5]Vitamin and mineral premix contained (g/kg of DM); 348.3 calcium carbonate (Pestell Minerals and Ingredients, New Hamburg, ON Canada), 283.7 zinc sulfate monohydrate (Pestell Minerals and Ingredients, New Hamburg, ON Canada), 103.1 cupric sulfate pentahydrate (Freeport-McMoRan Inc., Phoenix, AZ USA), 146.1 manganese sulfate monohydrate (Pestell Minerals and Ingredients, New Hamburg, ON Canada), 1.50 ethylenediamine dihydroiode (EDDI) (800 g EDDI/kg) (Pestell Minerals and Ingredients, New Hamburg, ON Canada), 50.4 selenium (10 g selenium/kg; Selenium Premix 1%, Pestell Minerals and Ingredients, New Hamburg, ON Canada), 0.80 cobalt carbonate (Pestell Minerals and Ingredients, New Hamburg, ON Canada), 17.7 vitamin A (1,000,000 IU/g) (Zhejiang Nvb Company Ltd., Zhejiang, China), 1.70 vitamin D_3 (500,000 IU/g) (Zhejiang Nvb Company Ltd., Zhejiang, China), and 47.3 vitamin E (all-rac-alpha tocopherol acetate) (500,000 IU/kg) (Zhejiang Nvb Company Ltd., Zhejiang, China).
[6]Les Mélasses Westway Inc., Brossard, QC Canada.
[7]Zhejiang Nvb Company Ltd., Zhejiang, China.

Table 9 Formulated chemical composition (% of DM, unless otherwise noted) of basal diet.

Chemical composition[1]	% of DM
DM, % of diet	40.48
OM	79.72
CP	13.07
Fat	2.99
NDF	53.11
ADF	38.12
Starch	16.54
TDN[2]	61.15
Ne_m[3], Mcal/kg	1.27
Ne_g[4], Mcal/kg	0.73

[1]Ration was formulated using beef cattle feed ration formulation software (National Research Council Nutrient Requirements of Beef Cattle Version 1.0.10 (March 2014), Washington, DC USA) using standard feed values and measured dry matters.
[2]Calculated using the equation TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).
[3]Net energy for maintenance.
[4]Net energy for gain.

Table 10 Enteric fermentation chamber door access schedule.

Chamber Number	Sampling Time (min of every h)	Best Time to Open Door (min of every h)
136	0-6	7
	30-36	37
140	6-13	14
	36-43	44
141	13-20	21
	43-50	51
142	20-27	28
	50-57	58

Table 11 Enteric fermentation chamber sampling schedule.

Chamber Number	Sampling Time (min of every h)	Gas	
		CO_2	CH_4
136	0	Exh	Int
	3	Int	Exh
140	6	Int	Exh
	9	Exh	Int
141	13	Exh	Int
	16	Int	Exh
142	20	Int	Exh
	23	Exh	Int
Reference	27	Nitrogen	
136	30	Exh	Int
	33	Int	Exh
140	36	Int	Exh
	39	Exh	Int
141	43	Exh	Int
	46	Int	Exh
142	50	Int	Exh
	53	Exh	Int
Reference	57	Nitrogen	

Table 12 Analyzed chemical composition, fatty acid profile, gross and digestible energy content, and physical characteristics of CON, OIL, NOP and NOP+OIL dietary treatments.

Item	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Chemical Composition[2]						
DM, %	35.31[b]	35.64[a]	33.97[c]	35.20[b]	0.08	0.002
OM, % of DM	91.01[b]	91.56[a]	91.02[b]	91.61[a]	0.10	0.0009
CP, % of DM	13.35[a]	12.58[b]	13.40[a]	12.63[b]	0.10	<0.0001
Fat, % of DM	3.24[b]	8.46[a]	3.08[b]	8.33[a]	0.15	<0.0001
NDF, % of DM	43.05[ab]	42.14[b]	43.94[a]	41.55[b]	0.52	0.03
ADF, % of DM	25.33[ab]	24.75[b]	26.10[a]	24.46[b]	0.34	0.02
Starch, % of DM	26.00[a]	24.55[bc]	25.25[ab]	23.76[c]	0.28	0.002
TDN[3], % of DM	72.80[ab]	73.34[a]	72.10[b]	73.59[a]	0.31	0.02
Fatty acid[4], mg of fatty acid/ g of sample						
SFA[5]	5.43[b]	8.59[a]	5.27[b]	8.45[a]	0.08	<0.0001
MUFA[6]	4.12[b]	37.01[a]	4.19[b]	36.59[a]	0.74	<0.0001
PUFA[7]	10.03[b]	24.95[a]	9.80[b]	24.77[a]	0.09	<0.0001
Total fatty acids	19.85[b]	70.53[a]	19.53[b]	69.89[a]	0.63	<0.0001
Fatty acid[4], % of fatty acid						
SFA[5]	27.66[a]	12.11[b]	27.34[a]	12.07[b]	0.94	<0.0001
MUFA[6]	20.78[b]	52.27[a]	21.59[b]	52.24[a]	0.51	<0.0001
PUFA[7]	50.92[a]	35.35[b]	50.41[a]	35.42[b]	0.43	<0.0001
GE, Mcal/kg	5.27[c]	5.57[a]	5.21[d]	5.52[b]	0.02	<0.0001
DE, Mcal/kg	3.56[a]	3.39[b]	3.47[ab]	3.40[b]	0.07	0.004

Analyzed chemical composition, fatty acid profile, and gross and digestible energy content of CON, OIL, NOP and NOP+OIL dietary treatments.

Item	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Particle Size[8]						
Upper Level, % > 19 mm	10.12	11.99	11.17	11.83	0.72	0.27
Middle Level, % 8 - 19 mm	64.02	64.68	64.63	65.37	0.52	0.35
Bottom Level, % 1.18 - 8 mm	23.77[a]	22.18[b]	22.34[b]	21.62[b]	0.39	0.005
Bottom Pan, % <1.18 mm	2.12[a]	1.18[b]	1.88[a]	1.19[b]	0.10	<0.0001

[a-c]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n= 8.
[3]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).
[4]n= 4.
[5]Total saturated fatty acids including C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0.
[6]Total monounsaturated fatty acids including C16:1 *trans*-3, C16:1 *cis*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C20:1 *cis*-11, C22:1 *cis*-13, and C24:1 *cis*-15.
[7]Total polyunsaturated fatty acids including C18:2 n-6, C18:3 n-3, C20:4 n-6, C20:2 n-6, and C22:2 n-6.
[8]Particle size was determined using the Penn State Particle Separator according to Kononoff et al. (2003); n= 16.

Table 13 Analyzed chemical composition and physical characteristics of barley silage.

Item	Barley Silage (Mean ± SD)
Chemical Composition[1]	
DM, %	31.10 ± 1.42
OM, % of DM	91.06 ± 0.29
CP, % of DM	11.97 ± 0.56
Fat, % of DM	3.10 ± 0.11
NDF, % of DM	47.44 ± 2.20
ADF, % of DM	28.14 ± 1.59
Starch, % of DM	22.99 ± 3.16
TDN[2], % of DM	70.25 ± 1.45
Particle Size[2]	
Upper Level, % > 19 mm	12.72 ± 4.43
Middle Level, % 8 - 19 mm	67.31 ± 3.38
Bottom Level, % 1.18 - 8 mm	19.10 ± 2.44
Bottom Pan, % <1.18 mm	0.87 ± 0.48

[1]n= 16.
[2]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).
[3]Particle size was determined using the Penn State Particle Separator according to Kononoff et al. (2003); n= 16.

Table 14 Analyzed chemical composition and physical characteristics of dry-rolled barley grain.

Item	Dry-Rolled Barley Grain (Mean ± SD)
Chemical Composition[1]	
DM, %	95.96 ± 1.15
OM, % of DM	97.46 ± 0.19
CP, % of DM	13.08 ± 0.63
Fat, % of DM	1.74 ± 0.12
NDF, % of DM	21.23 ± 1.39
ADF, % of DM	5.65 ± 0.59
Starch, % of DM	59.99 ± 1.64
TDN[2], % of DM	90.74 ± 0.54
Bushel Weight, lbs./ bu.	42.68 ± 2.02
PI[3], %	81.93 ± 0.98
Particle Size[4]	
Screen #2, % > 4.00 mm	14.06 ± 13.00
Screen #4, % 2.36 - 4.00 mm	76.81 ± 9.87
Screen #5, % 1.18 - 2.36 mm	7.91 ± 2.98
Screen #6, % 850 μm - 1.18 mm	0.44 ± 0.23
Bottom Pan, % <850 μm	0.78 ± 0.40

[1]n= 16.
[2]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).
[3]Processing index (measure of bushel weight after processing as a percentage of bushel weight before processing) was measured according to Rode and Beauchemin (1998); n=7.
[4]Particle size was determined using the Rotap apparatus; n= 7.

Table 15 Fatty acid profile of canola oil.

Item[1]	Fatty acid methyl esters (Mean ± SD)
Fatty acid, mg of fatty acid/ g of sample	
SFA[2]	68.01 ± 3.60
MUFA[3]	651.91 ± 13.54
PUFA[4]	309.44 ± 4.14
Total fatty acids	1029.35 ± 14.52
Fatty acid, % of fatty acid	
SFA[2]	6.60 ± 0.27
MUFA[3]	63.33 ± 0.45
PUFA[4]	30.07 ± 0.71

[1]n=4.
[2]Total saturated fatty acids including C14:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0.
[3]Total monounsaturated fatty acids including C16:1 *cis*-7, C16:1 *cis*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C20:1 *cis*-11, C22:1 *cis*-13, and C24:1 *cis*-15.
[4]Total polyunsaturated fatty acids including C18:2 n-6, C18:3 n-3, C20:2 n-6, and C22:2 n-6.

Table 16 Analyzed chemical composition of supplement mix.

Chemical Composition[2]	Supplement Mix[1] (Mean ± SD)
DM, %	97.45 ± 1.24
OM, % of DM	84.48 ± 1.78
CP, % of DM	34.39 ± 0.93
Fat, % of DM	2.98 ± 0.14
NDF, % of DM	24.78 ± 1.01
ADF, % of DM	16.19 ± 0.44
Starch, % of DM	11.82 ± 1.14
TDN[3], % of DM	81.13 ± 0.40

[1]Supplement Mix contained 66% canola meal, 2% urea, 6% limestone, 1% Vitamin and mineral premix (348.3 g/kg of DM calcium carbonate, 283.7 g/kg of DM zinc sulphate monohydrate, 103.1 g/kg of DM cupric sulphate pentahydrate, 146.1 g/kg of DM manganese suplhate monohydrate, 1.50 g/kg of DM ethylenediamine dihydroiode (EDDI) (800 g EDDI/kg), 50.4 g/kg of DM selenium, 0.80 g/kg of DM cobalt carbonate, 17.7 g/kg of DM vitamin A (1,000,000 IU/g), 1.70 mg/kg of DM vitamin D3 (500,000 IU/g), and 47.3 mg/kg of DM vitamin E (all-rac-alpha tocopherol acetate)(500,000 IU/kg)), 10% molasses, 15% ground barley grain, and 0.10% Vitamin E.

[2]n= 8.

[3]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).

Table 17 Analyzed chemical composition of treatment mixes.

	Treatment Mix[1] (Mean ± SD)	
Chemical Composition[2]	Placebo	NOP
DM, %	92.20 ± 0.74	90.90 ± 0.85
OM, % of DM	84.64 ± 2.08	86.20 ± 2.22
CP, % of DM	9.12 ± 0.49	8.78 ± 0.84
Fat, % of DM	26.49 ± 1.71	16.13 ± 1.48
NDF, % of DM	26.10 ± 6.75	23.28 ± 2.05
ADF, % of DM	6.90 ± 1.94	6.93 ± 1.23
Starch, % of DM	40.76 ± 1.88	37.31 ± 3.83
TDN[3], % of DM	89.60 ± 1.77	89.56 ± 1.12

[1]Treatment mixes contained 63.19% ground barley, 14.20% canola oil (Richardson International, Winnipeg, MB Canada), and 22.61% of either 8.85% 3-nitrooxypropanol on silicon dioxide and propylene glycol (NOP and NOP+OIL treatments) or a placebo mix containing silicon dioxide and propylene glycol (Training Period basal diet, CON, and OIL treatments) (DSM Nutritional Products Ltd., Kaiseraugst, Switzerland).

[2]n=4.

[3]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).

Table 18 Body weight and dry matter, nutrient, gross energy and digestible energy intakes of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

	Treatment[1]				SEM	*P*-value
Item	CON	OIL	NOP	NOP+OIL		
Body weight[2], kg	715.81[b]	732.18[a]	714.44[b]	733.57[a]	14.12	<0.0001
DMI, kg/d	7.19[b]	7.35[a]	7.08[c]	7.31[a]	0.03	<0.0001
Intake[3], kg/d (DM basis)						
OM	6.54[b]	6.70[a]	6.41[c]	6.66[a]	0.05	<0.0001
NDF	3.13[a]	3.09[ab]	3.04[bc]	2.98[c]	0.02	0.0003
ADF	1.85[a]	1.82[ab]	1.79[bc]	1.75[c]	0.02	0.001
Fat	0.25[c]	0.62[a]	0.23[d]	0.59[b]	0.008	<0.0001
Starch	1.86[a]	1.83[a]	1.76[b]	1.80[ab]	0.03	0.02
TDN[4]	5.21[b]	5.36[a]	5.11[c]	5.38[a]	0.04	<0.0001
GE intake[5], Mcal/ d	37.90[c]	40.78[a]	36.61[d]	40.13[b]	0.32	<0.0001
DE intake[6], Mcal/ d	25.81	25.33	24.67	25.08	0.49	0.27

[a-c]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=8.
[3]n=224.
[4]TDN (% DM)= 95.88 – (0.911 × ADF) (CVAS, 2009).
[5]Gross energy (Mcal/kg) × dry matter intake (kg/d).
[6]Digestible energy (Mcal/kg) × dry matter intake (kg/d).

Table 19 Apparent total tract digestibility of the CON, OIL, NOP and NOP+OIL dietary treatments.

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
DM, %	66.64[a]	60.83[b]	66.95[a]	60.62[b]	0.99	<0.0001
OM, %	68.37[a]	62.42[b]	68.73[a]	61.96[b]	0.97	<0.0001
CP, %	67.54	67.17	69.62	68.10	0.81	0.06
NDF, %	58.97[a]	48.28[b]	60.96[a]	46.82[b]	1.25	<0.0001
ADF, %	58.05[a]	47.18[b]	60.02[a]	45.75[b]	1.35	<0.0001
Starch, %	96.42	96.58	96.81	96.71	0.23	0.15

[a-c]Least squares means within a row with different superscripts differ (P < 0.05).

[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.

[2]n=16. To calculate the apparent total tract digestibility (percent of nutrient digested), the following equation was used: $\% ND = \frac{NC - \mathrm{NF}}{NC} \times 100$ where *ND* is nutrient digestion (%), *NC* is nutrient consumed (kg), and *NF* is nutrient in feces (kg) (Fahey et al., 1994; Schneider and Flatt, 1975).

Table 20 Minimum, mean, maximum, and range of ruminal diurnal pH of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

	Treatment[1]					
Item	CON	OIL	NOP	NOP+OIL	SEM	*P*-value
Minimum	5.76[b]	5.66[bc]	5.91[a]	5.61[c]	0.05	<0.0001
Mean	6.49[c]	6.53[b]	6.57[a]	6.48[c]	0.05	<0.0001
Maximum	7.18	7.28	7.16	7.19	0.05	0.25
Range[2]	1.43[bc]	1.63[a]	1.25[c]	1.57[ab]	0.06	0.0005

[a-c]Least squares means within a row with different superscripts differ (P < 0.05).

[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.

[2]Maximum pH - minimum pH.

Table 21 Dissolved hydrogen, NH_3-N, and VFA in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
aqH_2, µmol/L	7.34^{b}	11.02^{b}	71.60^{a}	66.12^{a}	7.69	<0.0001
NH_3-N, m*M*	5.21^{a}	4.33^{b}	4.70^{ab}	3.71^{c}	0.24	<0.0001
Total VFA, m*M*	101.32^{a}	94.78^{b}	94.75^{b}	88.26^{c}	3.13	<0.0001
VFA, m*M*						
Acetate	63.72^{a}	55.47^{b}	52.26^{c}	47.35^{d}	1.18	<0.0001
Propionate	18.03^{c}	19.89^{a}	19.45^{ab}	18.66^{bc}	0.68	0.001
Iso-Butyrate	1.17^{a}	1.14^{b}	1.07^{b}	1.00^{b}	0.04	<0.0001
Butyrate	13.08^{c}	12.59^{c}	16.32^{a}	14.90^{b}	0.96	<0.0001
Iso-Valerate	2.32^{b}	2.73^{a}	2.01^{c}	2.85^{a}	0.22	<0.0001
Valerate	1.98^{b}	2.15^{b}	2.49^{a}	2.55^{a}	0.19	<0.0001
Caproate	1.01^{ab}	0.81^{c}	1.15^{a}	0.94^{bc}	0.01	0.0007
VFA, mol/100 mol						
Acetate	64.27^{a}	60.36^{b}	56.93^{c}	55.94^{c}	0.79	<0.0001
Propionate	17.55^{b}	20.43^{a}	20.49^{a}	20.76^{a}	0.44	<0.0001
Iso-Butyrate	1.18	1.24	1.16	1.19	0.04	0.12
Butyrate	12.08^{b}	12.39^{b}	15.87^{a}	15.40^{a}	0.62	<0.0001
Iso-Valerate	2.25^{c}	2.79^{b}	2.13^{c}	3.18^{a}	0.29	<0.0001
Valerate	1.80^{d}	2.04^{c}	2.38^{b}	2.58^{a}	0.13	<0.0001
Caproate	0.88^{bc}	0.75^{c}	1.04^{a}	0.95^{ab}	0.08	0.0005

Dissolved hydrogen, NH_3-N, and VFA profile in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments (continued).

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Acetate : Propionate	3.72[a]	3.09[b]	2.83[c]	2.80[c]	0.09	<0.0001
Acetate + Butyrate : Propionate	4.41[a]	3.70[b]	3.63[b]	3.55[b]	0.10	<0.0001

[a-d]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=80

Table 22 Fatty acid profile of rumen contents of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments 3 h after feeding.

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Fatty acid, mg of fatty acid/ g of sample						
SFA[3]	14.80[b]	37.74[a]	16.47[b]	39.88[a]	1.38	<0.0001
BCFA[4]	0.95[ab]	0.89[b]	1.09[a]	0.85[b]	0.07	0.01
trans-MUFA[5]	1.97[b]	8.02[a]	2.28[b]	7.24[a]	0.35	<0.0001
cis-MUFA[6]	2.06[b]	8.22[a]	1.98[b]	7.49[a]	1.17	0.002
PUFA[7]	3.37[a]	2.60[b]	3.04[a]	2.68[b]	0.44	0.02
CLA[8]	0.19	0.29	0.19	0.24	0.03	0.23
CLNA[9]	0.04	0.07	0.05	0.07	0.01	0.08
Atypical dienes[10]	0.33[b]	1.06[a]	0.33[b]	1.09[a]	0.09	<0.0001
Other[11]	0.09	0.08	0.07	0.07	0.007	0.35
Total fatty acids	23.80[b]	58.96[a]	25.51[b]	59.61[a]	1.31	<0.0001
Fatty acid, % of fatty acid						
SFA[3]	62.23	64.31	64.14	67.05	1.89	0.31
BCFA[4]	4.02[a]	1.51[b]	4.23[a]	1.43[b]	0.20	<0.0001
trans-MUFA[5]	8.26[c]	13.56[a]	8.97[c]	12.11[b]	0.51	<0.0001
cis-MUFA[6]	8.69[b]	13.74[a]	7.89[b]	12.50[a]	0.99	0.0006
PUFA[7]	14.09[a]	4.37[b]	12.18[a]	4.47[b]	1.48	<0.0001
CLA[8]	0.80[a]	0.49[ab]	0.76[a]	0.40[b]	0.12	0.03
CLNA[9]	0.16	0.12	0.19	0.11	0.03	0.33

Fatty acid concentration of rumen contents of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments 3 h after feeding.

Item[2],	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Fatty acid[3], % of fatty acid						
Atypical dienes[10]	1.39[ab]	1.77[a]	1.33[b]	1.82[a]	0.19	0.02
Other[11]	0.38[a]	0.13[b]	0.29[a]	0.12[b]	0.03	0.0001

[a-b]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=8.
[3]Total saturated fatty acids including C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0.
[4]Total branch chain fatty acids including C15:0iso, C15:0anteiso, C16:0iso, C17:0iso, C17:0anteiso, and C18:0iso.
[5]Total *trans*-monounsaturated fatty acids including C16:1 *trans*-9, C18:1 *trans*-4, C18:1 *trans*-5, C18:1 *trans*-6, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13, C18:1 *trans*-14, C18:1 *trans*-15, and C18:1 *trans*-16.
[6]Total *cis*-monounsaturated fatty acids including C16:1 *cis*-7, C16:1 *cis*-9, C17:1 *cis*-9, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *cis*-14, C18:1 *cis*-15, C20:1 *cis*-11, C22:1 *cis*-13, and C24:1 *cis*-15.
[7] Total polyunsaturated fatty acids including C18:2 n-6, C18:3 n-3, C20:4 n-6, C20:2 n-6, and C22:2 n-6.
[8]Total conjugated linoleic fatty acids including C18:2 *cis*-9 *trans*-11, C18:2 *trans*-7 *cis*-9, C18:2 *trans*-10 *cis*-12, C18:2 *trans*-11 *cis*-13, C18:2 *trans*-7 *trans*-9, and C18:2 *trans*-10 *trans*-12.
[9]Total conjugated linolenic fatty acids includes C18:3 *cis*-9 *trans*-11 *cis*-15.
[10]Total atypical diene fatty acids including C18:2 *trans*-11 *trans*-15, C18:2 *trans*-9 *trans*-12, C18:2 *cis*-9 *trans*-12, C18:2 *cis*-9 *trans*-13, C18:2 *cis*-9 *trans*-14, C18:2 *cis*-9 *trans*-15, C18:2 *cis*-9 *trans*-16, C18:2 *trans*-9 *cis*-2, C18:2 *trans*-9 *cis*-15, C18:2 *trans*-10 *cis*-15, C18:2 *cis*-9 *cis*-15, and C18:2 *cis*-12 *trans*-15.
[11]Other fatty acids includes *cis*-11 cyclohexyl-C17:0.

Table 23 Protozoal populations in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Protozoa, mL of rumen fluid						
Holotrichs						
Isotricha spp.	1.25×10^{2}	0.00	5.00×10^{2}	0.00	1.84×10^{2}	0.10
Dasytricha spp.	8.75×10^{2ab}	0.00^{b}	1.33×10^{3a}	0.00^{b}	4.36×10^{2}	0.007
Entodiniomorphs						
Entodinium spp.	3.97×10^{5a}	2.70×10^{4b}	4.41×10^{5a}	6.15×10^{4b}	4.72×10^{4}	<0.0001
Ostracodinium spp.	7.50×10^{2a}	0.00^{b}	1.08×10^{3a}	0.00^{b}	3.12×10^{2}	0.002
Metadinium spp.	7.50×10^{2a}	0.00^{b}	7.92×10^{2a}	4.20×10^{1b}	2.47×10^{2}	0.006
Osphyoscolex spp.	8.75×10^{2a}	0.00^{b}	1.38×10^{3a}	0.00^{b}	4.15×10^{2}	0.0009
Total, mL of rumen fluid	4.01×10^{5a}	2.70×10^{4b}	4.46×10^{5a}	6.15×10^{4b}	1.03×10^{5}	<0.0001

[a-b]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=48.

Table 24 Rumen volume and rumen content dry matter of animals 4 h after feeding.

Animal	Rumen Volume (wet basis, kg)	Rumen Contents DM (%)	Rumen Volume (dry basis, kg)	Rumen Volume (wet basis, % of BW)	Rumen Volume (dry basis, % of BW)
2	82.27	11.68	9.61	10.47	1.22
6	101.23	10.12	10.24	13.64	1.38
8	65.68	12.90	8.47	10.03	1.29
9	85.31	12.05	10.28	11.69	1.41
10	88.15	13.04	11.50	11.41	1.49
11	90.62	10.76	9.75	12.16	1.31
12	97.09	11.29	10.96	12.81	1.45
21	74.65	11.65	8.70	10.28	1.20
Mean ± SD	85.63 ± 11.56	11.69 ± 0.99	9.94 ± 1.04	11.56 ± 1.28	1.34 ± 0.11

Table 25 Enteric fermentation measurements (CH_4, H_2, and CO_2 emissions) of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

Item	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
CH_4 emissions						
CH_4, g/d	191.07[a]	140.24[b]	124.94[c]	93.24[d]	9.31	<0.0001
CH_4, g/kg of DMI	26.24[a]	19.57[b]	17.88[b]	12.69[c]	1.23	<0.0001
CH_4, g/kg of dDMI[3]	41.37[a]	29.99[b]	28.09[b]	18.81[c]	2.45	<0.0001
CH_4, % of GE Intake[4]	5.94[a]	4.21[b]	4.11[b]	2.75[c]	0.27	<0.0001
CH_4, g/kg of BW	0.25[a]	0.20[b]	0.17[c]	0.13[d]	0.009	<0.0001
H_2 emissions						
H_2, g/d	0.00[c]	0.09[c]	1.11[a]	0.61[b]	0.12	0.001
H_2, g/kg of DMI	0.00[c]	0.01[c]	0.16[a]	0.08[b]	0.02	0.001
H_2, g/kg of dDMI[3]	0.01[c]	0.01[c]	0.28[a]	0.20[b]	0.02	<0.0001
H_2, % of GE Intake[4]	0.00[c]	0.01[c]	0.16[a]	0.08[b]	0.02	0.0002
H_2, g/kg of BW	0.000[c]	0.000[c]	0.002[a]	0.001[b]	0.0002	<0.0001
CO_2 emissions						
CO_2, kg/d	8.23	8.02	8.24	8.35	0.45	0.30
CO_2, kg/kg of DMI	1.12	1.09	1.15	1.13	0.05	0.19
CO_2, kg/kg of BW	0.01	0.01	0.01	0.01	0.0006	0.10

[a-c]Least squares means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.

Enteric fermentation measurements (CH_4, H_2, and CO_2 emissions) of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments (continued).

[2]n=8.
[3]Digestible dry matter intake calculated from DMI (kg) in the chambers and digestibilities (%) of the dietary treatments (CON = 66.64%; OIL = 60.83%, NOP = 66.95%, and NOP+OIL = 60.62%).
[4]Gross energy intake calculated from DMI (kg) in the chambers and GE content (Mcal/kg) of the dietary treatments.

Table 26 Total volume and components of urine of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

Item[2]	Treatment[1]				SEM	*P*-value
	CON	OIL	NOP	NOP+OIL		
Total volume, L/d	11.95[b]	12.27[b]	14.27[a]	12.33[b]	2.60	0.05
Specific gravity	1.013[a]	1.005[b]	1.003[b]	1.004[b]	0.003	0.04
pH	7.92	7.83	8.00	7.96	0.08	0.44
Protein, mg/dL	30.21	35.42	30.21	32.29	6.33	0.88
Ketones, mg/dL	5.87	4.82	3.55	1.54	2.53	0.65
Erythocytes, number of cells/ µL	178.13	222.92	192.71	213.54	18.72	0.11
Allantoin, mg/L	376.11[ab]	420.76[a]	321.16[b]	397.51[a]	50.47	0.008
Urea, g/L	4.31	5.23	4.10	4.56	7.04	0.20
Ammonia-N, m*M*	4.23[a]	3.30[b]	4.19[a]	3.20[b]	0.46	0.002
Uric acid, mg/dL	8.35[b]	7.76[b]	9.51[a]	7.79[b]	0.57	0.008
Nitrogen, g/L	7.16	7.91	6.59	7.19	1.05	0.51

[a-c]Least squares means within a row with different superscripts differ (P < 0.05).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=32. Glucose, urobilinogen, bilirubin, nitrite, and leukocytes were negative for all treatments.

Table 27 Total weight, nutrient output, chemical composition, gross energy content, and pH of feces of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.

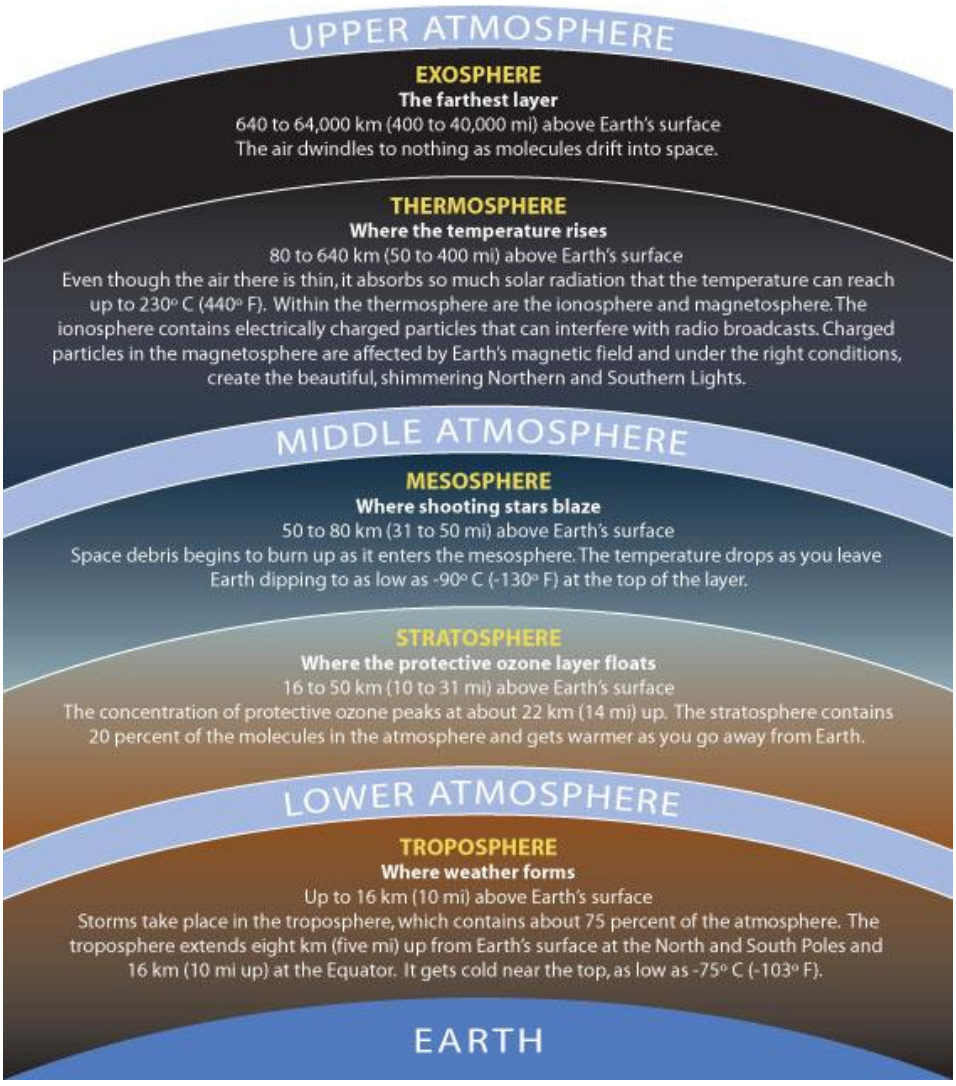
Item	Treatment[1]				SEM	P-value
	CON	OIL	NOP	NOP+OIL		
Total weight, kg/d						
Wet	12.30[b]	13.61[a]	10.76[c]	13.46[a]	0.31	<0.0001
Dry	2.24[b]	2.63[a]	2.09[c]	2.64[a]	0.06	<0.0001
Output, kg/d (DM basis)[2]						
OM	2.01[b]	2.45[a]	1.97[b]	2.46[a]	0.05	<0.0001
CP	0.32[a]	0.31[ab]	0.28[c]	0.29[bc]	0.007	0.001
NDF	1.44[b]	1.83[a]	1.41[b]	1.79[a]	0.05	<0.0001
ADF	0.85[b]	1.11[a]	0.84[b]	1.08[a]	0.03	<0.0001
Starch	0.06[a]	0.06[a]	0.06[b]	0.06[b]	0.004	0.003
Chemical Composition[2]						
DM, %	19.11[b]	20.02[a]	19.78[a]	19.57[ab]	0.36	0.01
OM, % of DM	84.63[d]	86.93[b]	85.45[c]	87.58[a]	0.40	<0.0001
CP, % of DM	14.54[a]	12.10[b]	14.14[a]	11.60[c]	0.19	<0.0001
NDF, % of DM	59.24[b]	62.87[a]	58.77[b]	62.96[a]	0.52	<0.0001
ADF, % of DM	36.89[b]	38.82[a]	35.99[b]	38.47[a]	0.47	<0.0001
Starch, % of DM	3.04[a]	2.53[bc]	2.76[ab]	2.35[c]	0.14	<0.0001
GE, Mcal/kg	5.22[b]	5.61[a]	5.17[b]	5.55[a]	0.06	<0.0001
GE Output[3], Mcal/d	12.62[b]	16.14[a]	12.46[b]	15.97[a]	0.50	<0.0001
pH	7.54[a]	7.53[a]	7.56[a]	7.30[b]	0.05	<0.0001

Total weight, nutrient output, chemical composition, gross energy content, and pH of feces of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments (continued).

[a-d]Least squares means within a row with different superscripts differ ($P < 0.05$).
[1]Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
[2]n=32.
[3]Gross energy (Mcal/kg) × fecal output (kg/d).

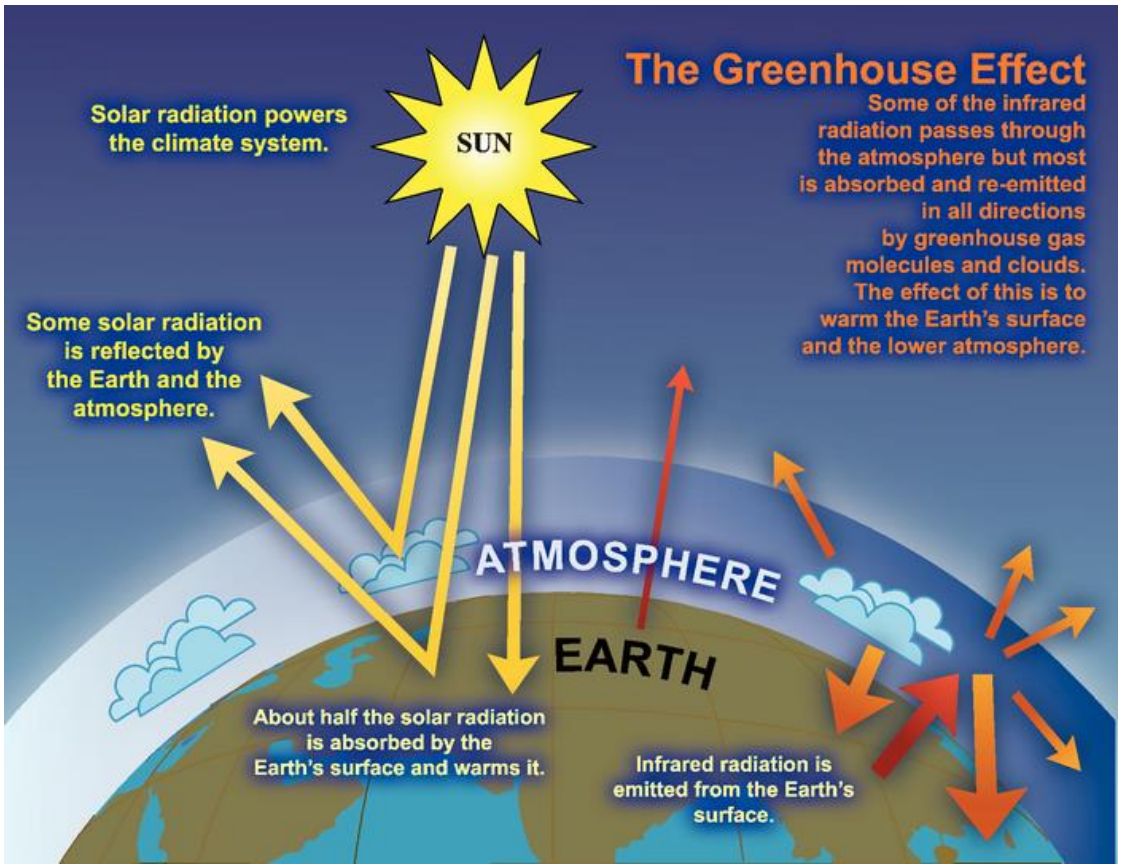
FIGURES

Figure 1 The five main layers of the atmosphere.



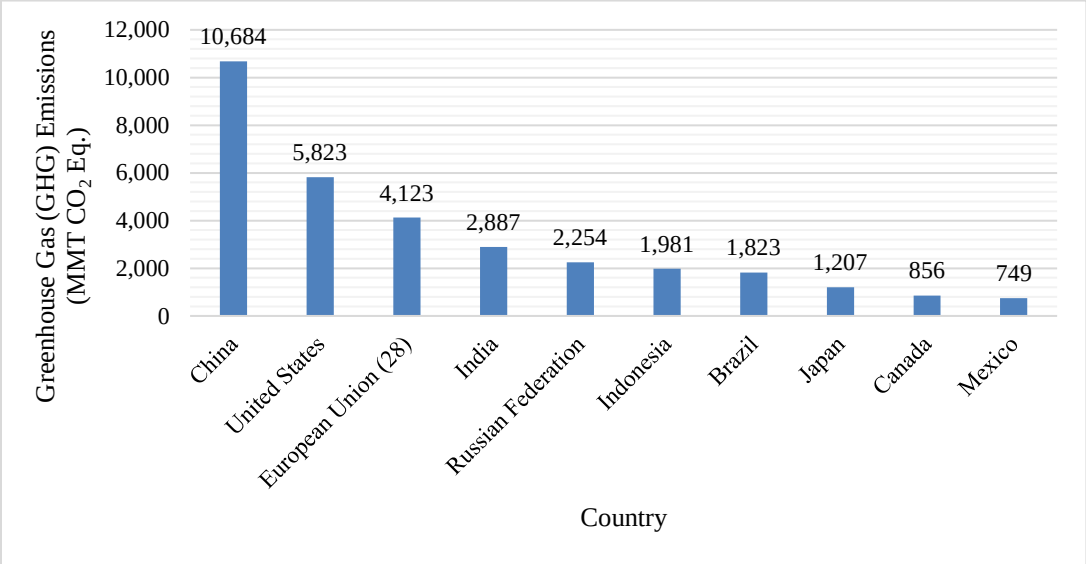
(Smithsonian, 2015).

Figure 2 The Greenhouse Effect.



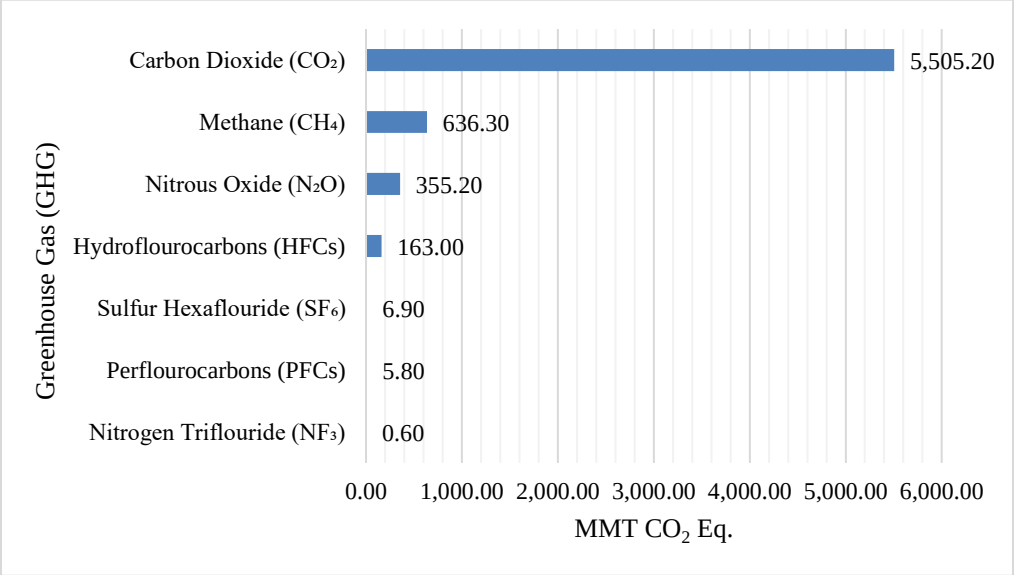
(IPCC, 2007).

Figure 3 Top 10 net anthropogenic GHG emission producing countries including land-use change and forestry in 2012.



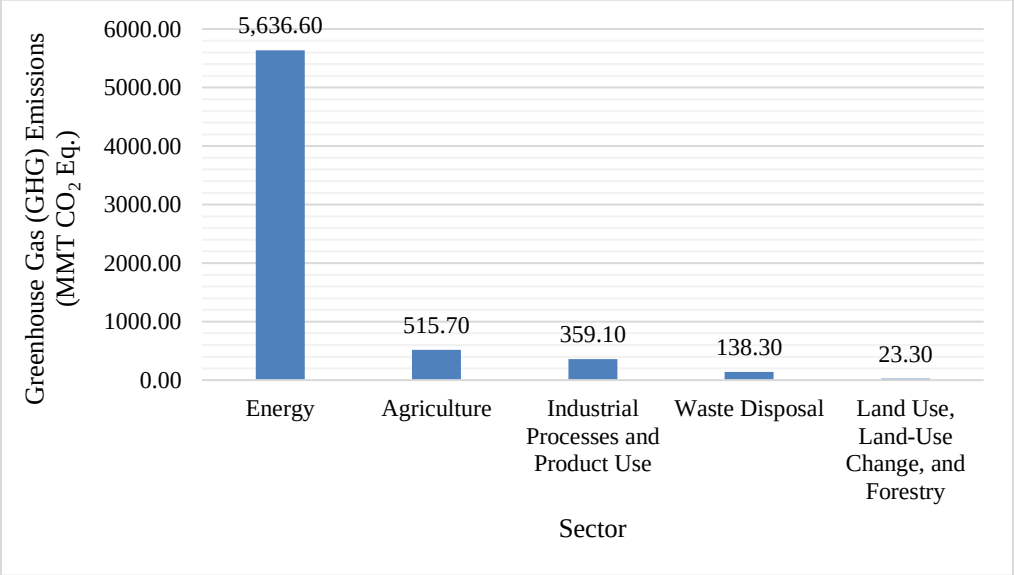
(World Resources Institute, 2015).

Figure 4 U.S. total anthropogenic GHG emissions in 2013.



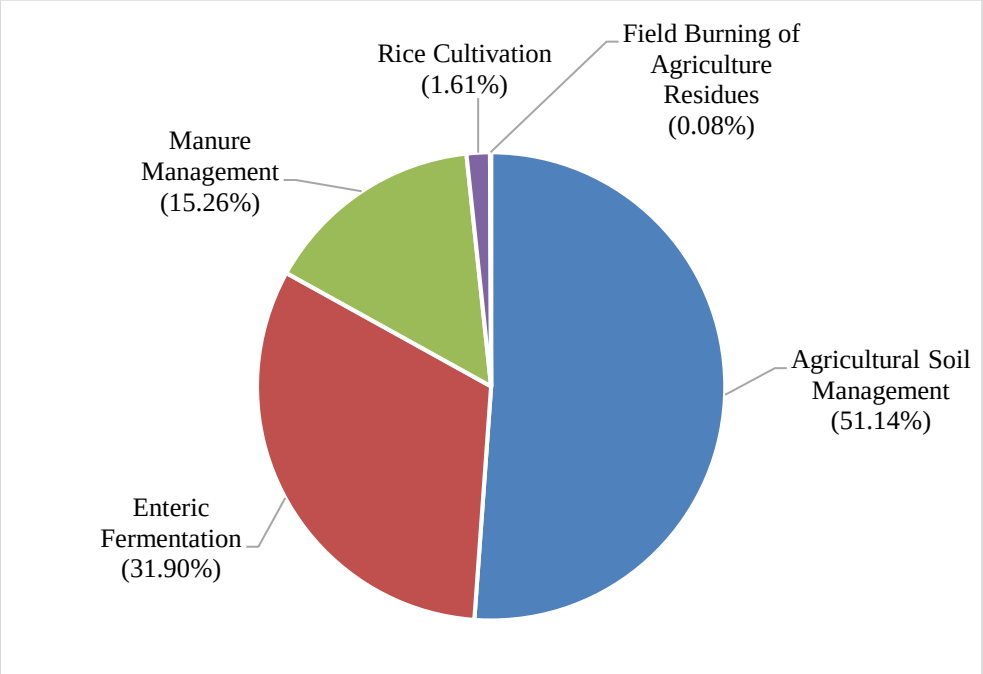
(IPCC, 2013; EPA, 2015).

Figure 5 U.S. total anthropogenic GHG emissions by sector in 2013.



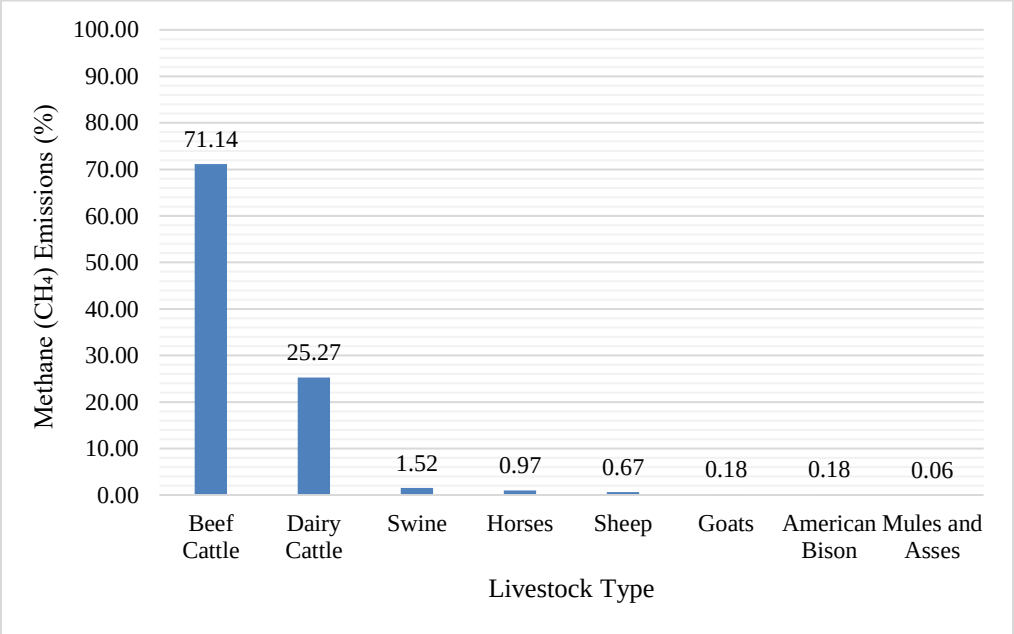
(IPCC, 2013; EPA, 2015).

Figure 6 U.S. total anthropogenic GHG emissions from agriculture in 2013.



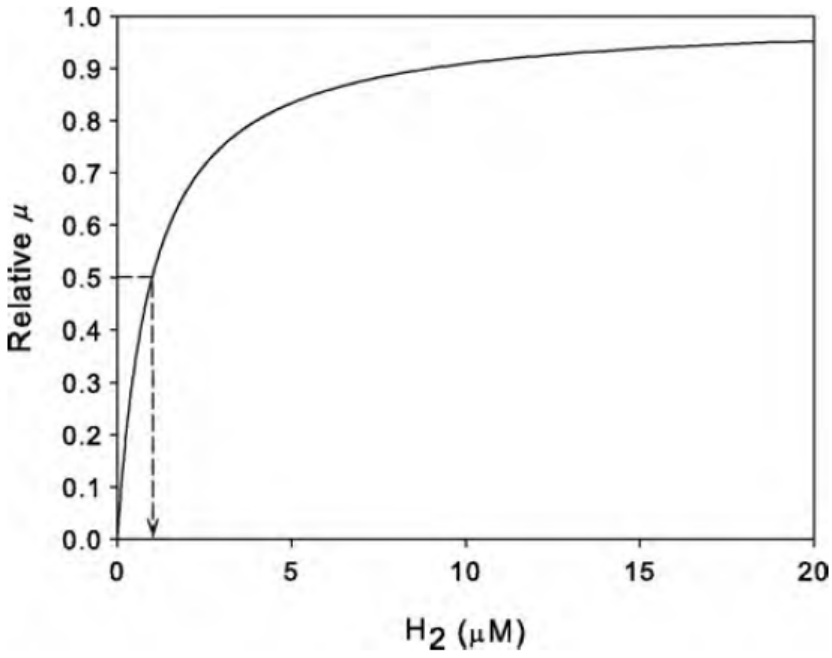
(IPCC, 2013; EPA, 2015).

Figure 7 Methane emissions from enteric fermentation of the Agriculture Sector in 2013.



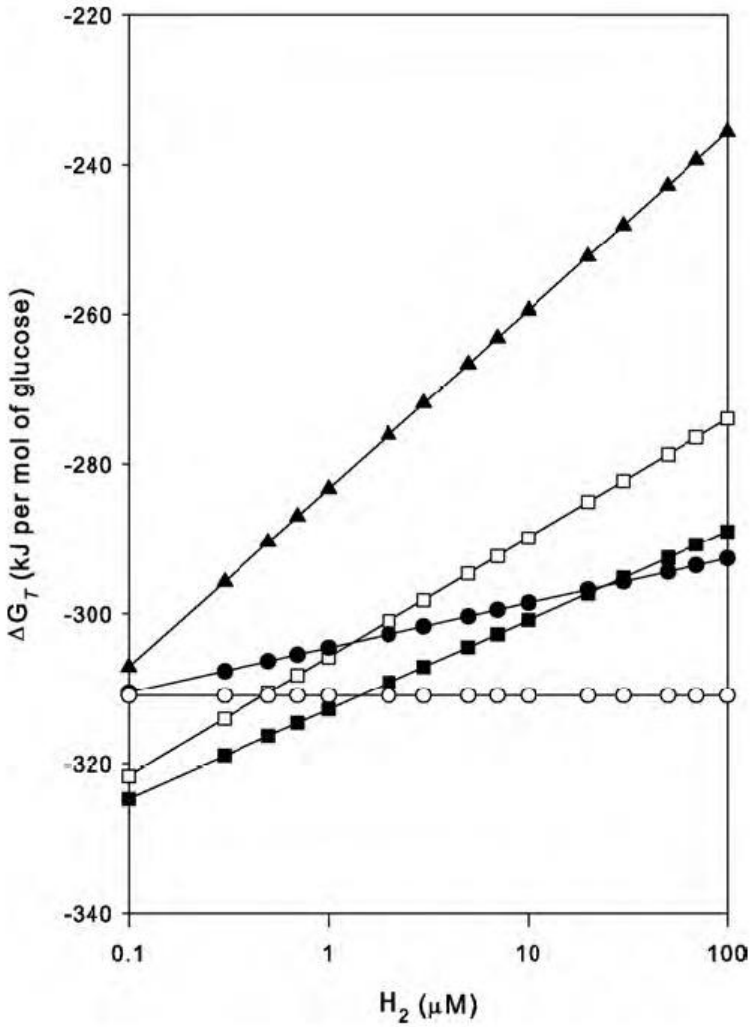
(IPCC, 2013; EPA, 2015).

Figure 8 The Monod relationship for the growth of a microorganism (methanogen) with its energy source (H_2).



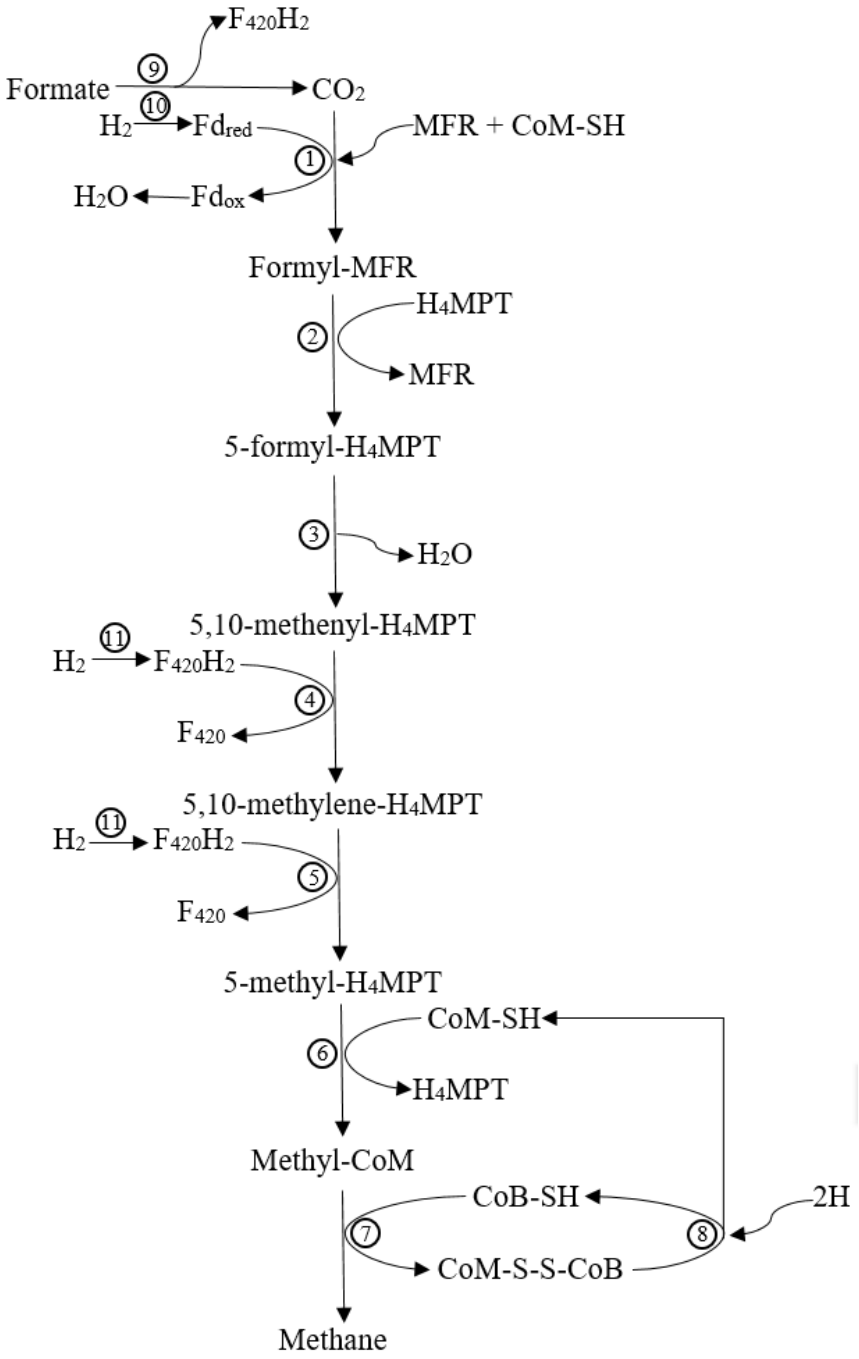
(Janssen, 2010).

Figure 9 Gibbs free energy changes (ΔG_T) for fermentation of glucose at different H_2 concentrations.



(Janssen, 2010). ▲ = glucose → 2acetate + 4H_2; ■ = glucose → butyrate + 2H_2; ● = glucose → acetate + propionate + H_2; □ = glucose → 0.66acetate + 0.66butyrate + 2.66H_2; and ○ = glucose → 0.66acetate + 1.33propionate.

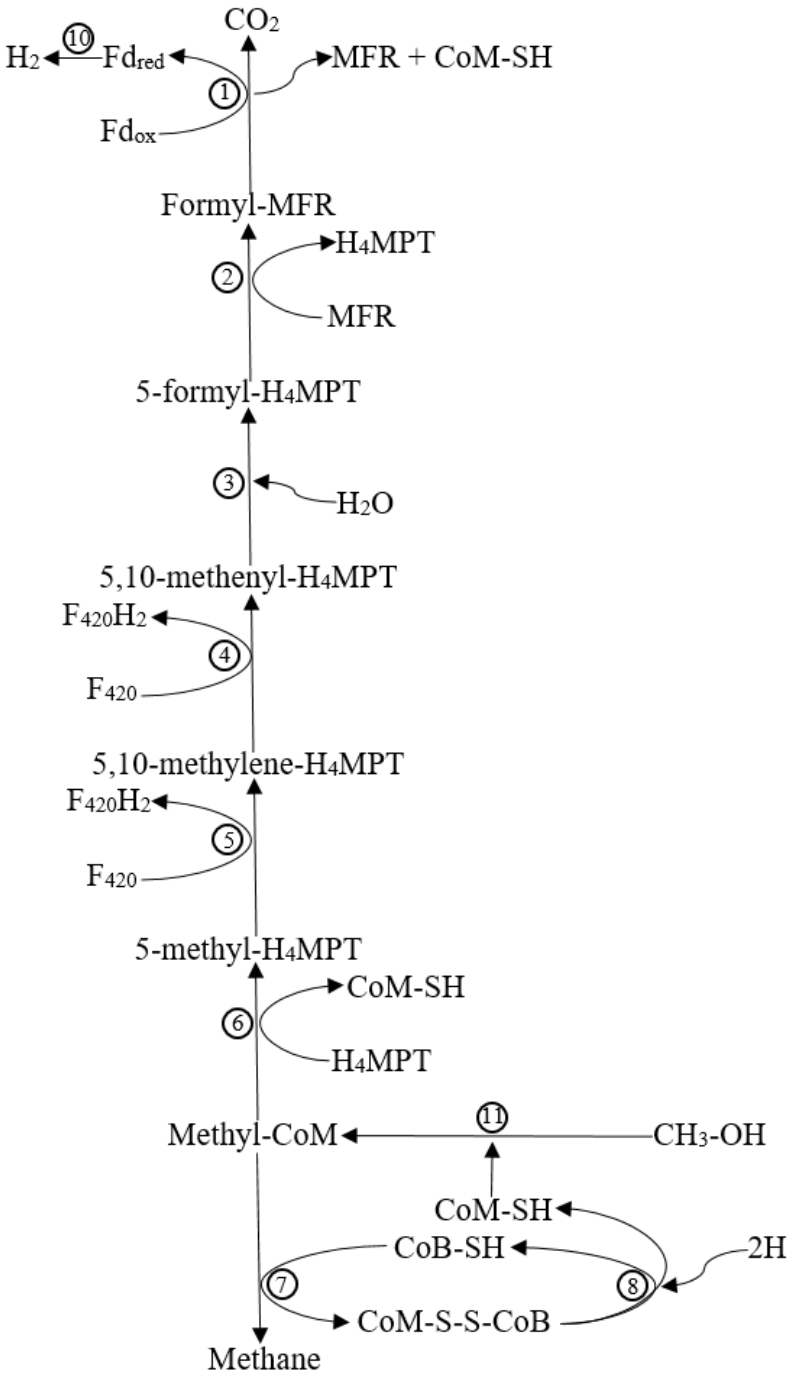
Figure 10 The hydrogenotrophic pathway.



The hydrogenotrophic pathway (continued).

(modified from Liu and Whitman, 2008 and Hoffman, 2014.)
Abbreviations: Fd_{red} = reduced form of ferredoxin; Fd_{ox} = oxidized form of ferredoxin; $F_{420}H_2$ = reduced form coenzyme F_{420}; MFR = methanofuran; H_4MPT = tetrahydromethan-opterin; CoM-SH =coenzyme M; CoB-SH = coenzyme B; CoM-S-S-CoB = heterodisulfide of CoM and CoB.
Enzymes: 1. formyl-MFR dehydrogenase (Fmd); 2. formyl-MFR:H_4MPT formyltransferase (Ftr); 3. methenyl-H4MPT cyclohydrolase (Mch); 4. methylene-H_4MPT dehydrogenase (Hmd); 5. methylene-H_4MPT reductase (Mer); 6. methyl-H_4MPT:CoM-SH methyltransferase (Mtr); 7. methyl-CoM reductase (Mcr); 8. heterodisulfide reductase (Hdr); 9. formate dehydrogenase (Fdh); 10. energy conserving hydrogenase (Ech); and 11. F_{420}-reducing hydrogenases.

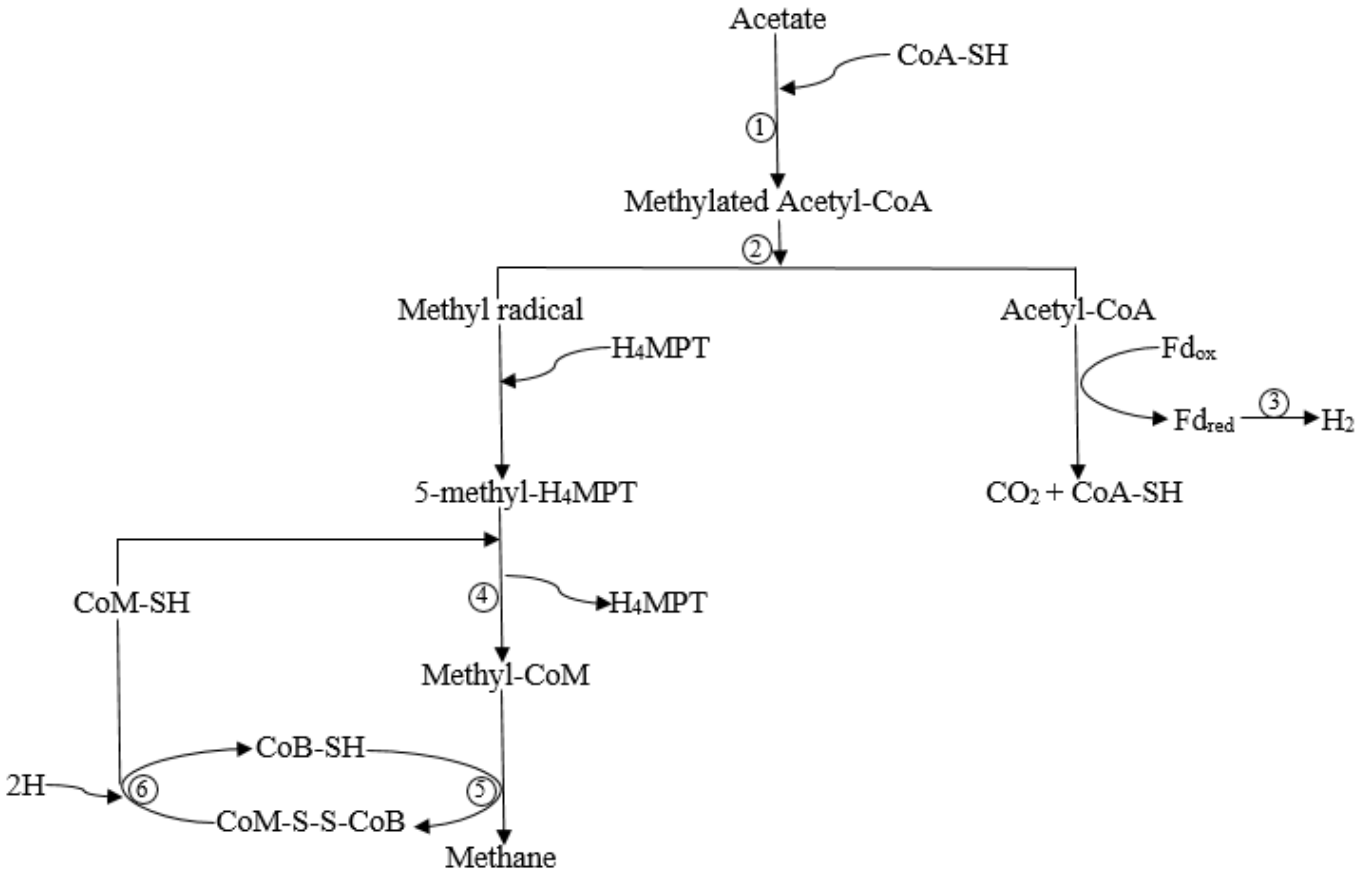
Figure 11 The methylotrophic pathway.



The methylotrophic pathway (continued).

(modified from Liu and Whitman, 2008 and Hoffman, 2014.) Abbreviations: Fd_{red} = reduced form of ferredoxin; Fd_{ox} = oxidized form of ferredoxin; $F_{420}H_2$ = reduced form coenzyme F_{420}; MFR = methanofuran; H_4MPT = tetrahydromethan-opterin; CoM-SH =coenzyme M; CoB-SH = coenzyme B; CoM-S-S-CoB = heterodisulfide of CoM and CoB.
Enzymes: 1. formyl-MFR dehydrogenase (Fmd); 2. formyl-MFR:H_4MPT formyltransferase (Ftr); 3. methenyl-H4MPT cyclohydrolase (Mch); 4. methylene-H_4MPT dehydrogenase (Hmd); 5. methylene-H_4MPT reductase (Mer); 6. methyl-H_4MPT:CoM-SH methyltransferase (Mtr); 7. methyl-CoM reductase (Mcr); 8. heterodisulfide reductase (Hdr); 9. formate dehydrogenase (Fdh); 10. energy conserving hydrogenase (Ech); and 11. methyltransferase

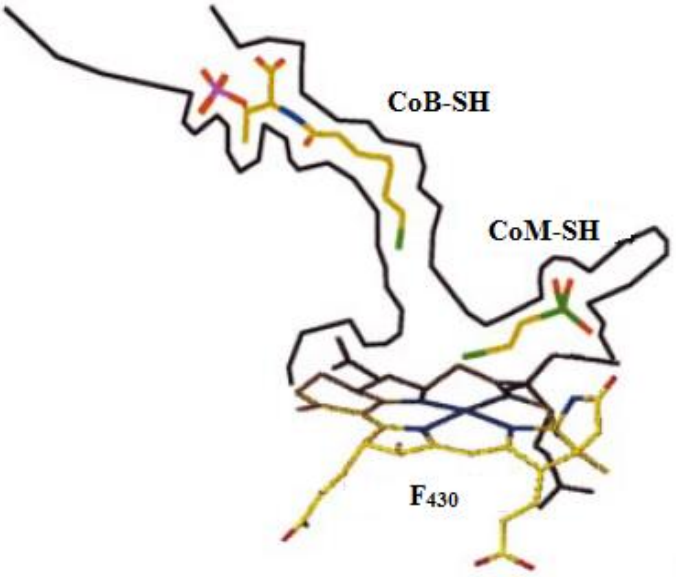
Figure 12 The aceticlastic pathway.



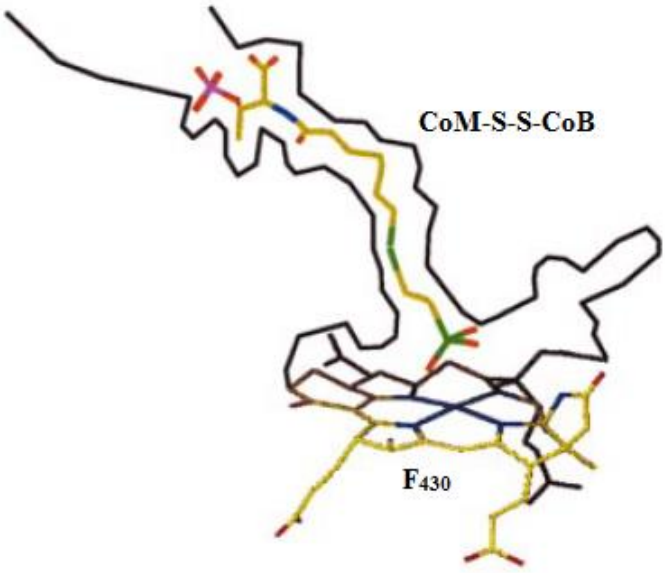
The aceticlastic pathway (continued).

(modified from Liu and Whitman, 2008 and Hoffman, 2014.) Abbreviations: Fd_{red} = reduced form of ferredoxin; Fd_{ox} = oxidized form of ferredoxin; H_4MPT = tetrahydromethanopterin; CoM-SH = coenzyme M; CoB-SH = coenzyme B; CoM-S-S-CoB = heterodisulfide of CoM and CoB; CoA-SH = coenzyme A.
Enzymes: 1. acetate kinase- phosphotransacetylase (AK-PTA); 2. carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), 3. Energy conserving hydrogenase (Ech); 4. methyl-H_4MPT:CoM-SH methyltransferase (Mtr); 5. methyl-CoM reductase (Mcr); and 6. heterodisulfide reductase (Hdr).

Figure 13 Schematic drawing of methyl-coenzyme M reductase (A) before and (B) after coenzyme M and coenzyme B binding.



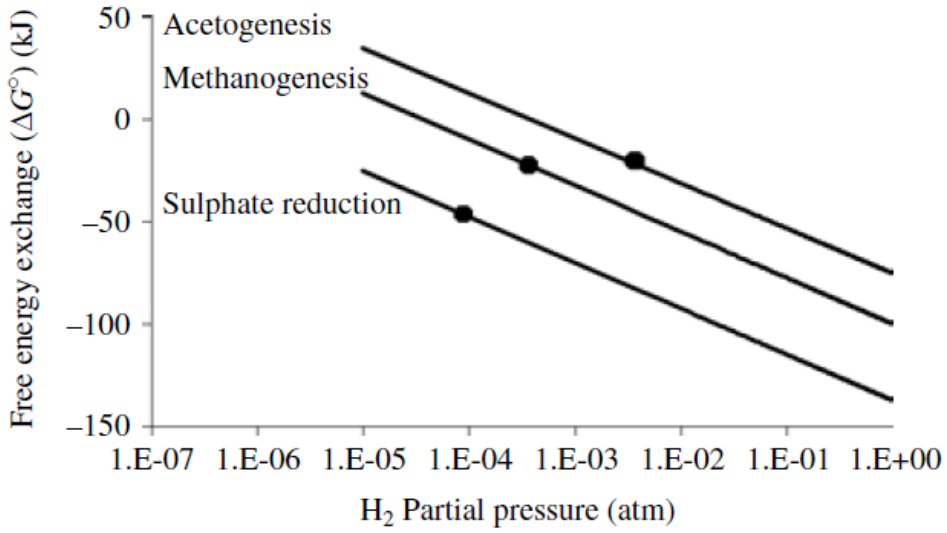
A.



B.

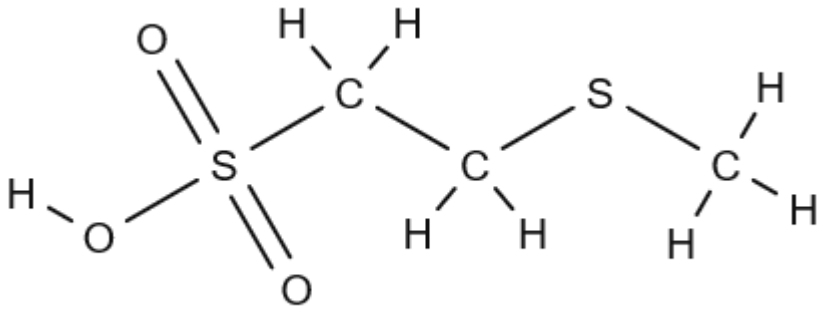
(modified from Grabarse et al., 2001).

Figure 14 Relationship between the free energy released from a reaction ($\Delta G°$) and the H_2 partial pressure (atm) of the system for methanogens, acetogens and sulphate-reducing bacteria. Solid dots represent the theshold H_2 partial pressure required for the reaction to occur.



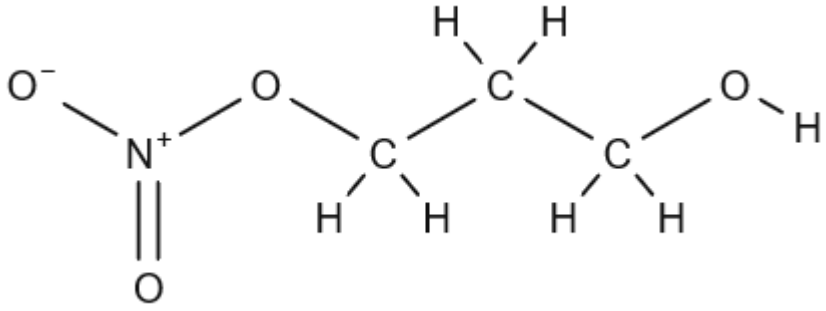
(Ellis et al., 2008).

Figure 15 Structure of methyl-coenzyme M.



(Bergwerf, 2015).

Figure 16 Structure of 3-nitrooxypropanol.



(Bergwerf, 2015).

Figure 17 Homemade rumen fluid sampling filter.

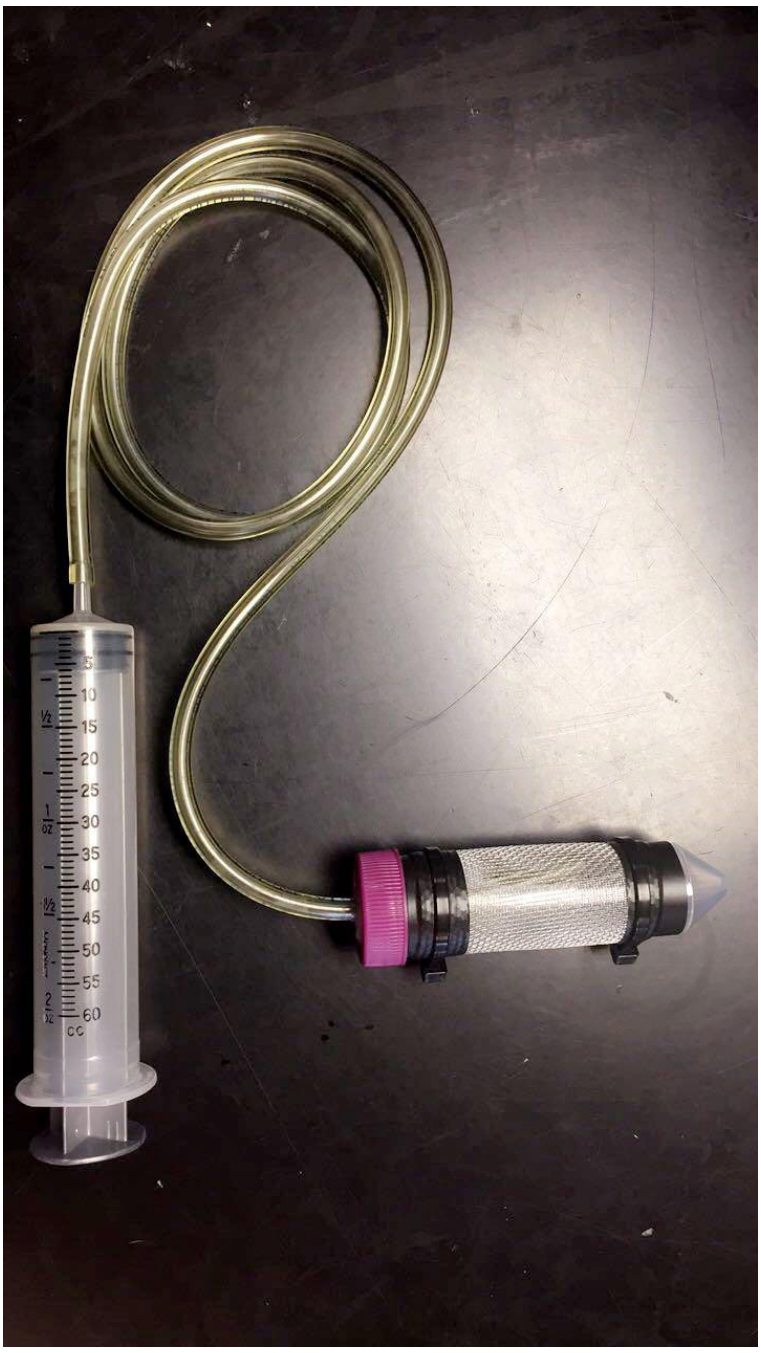
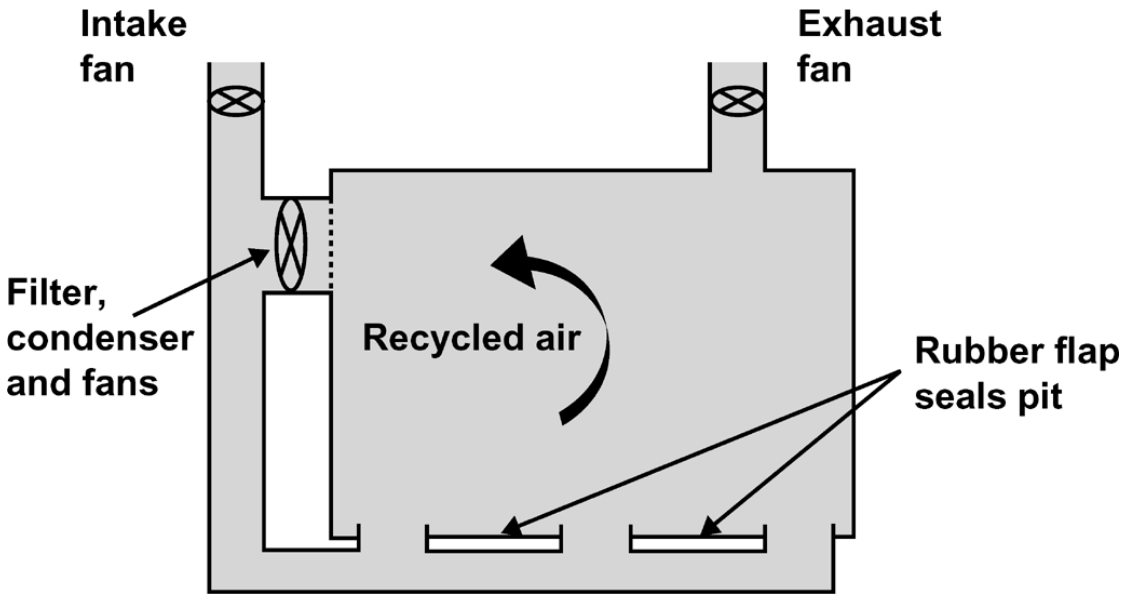
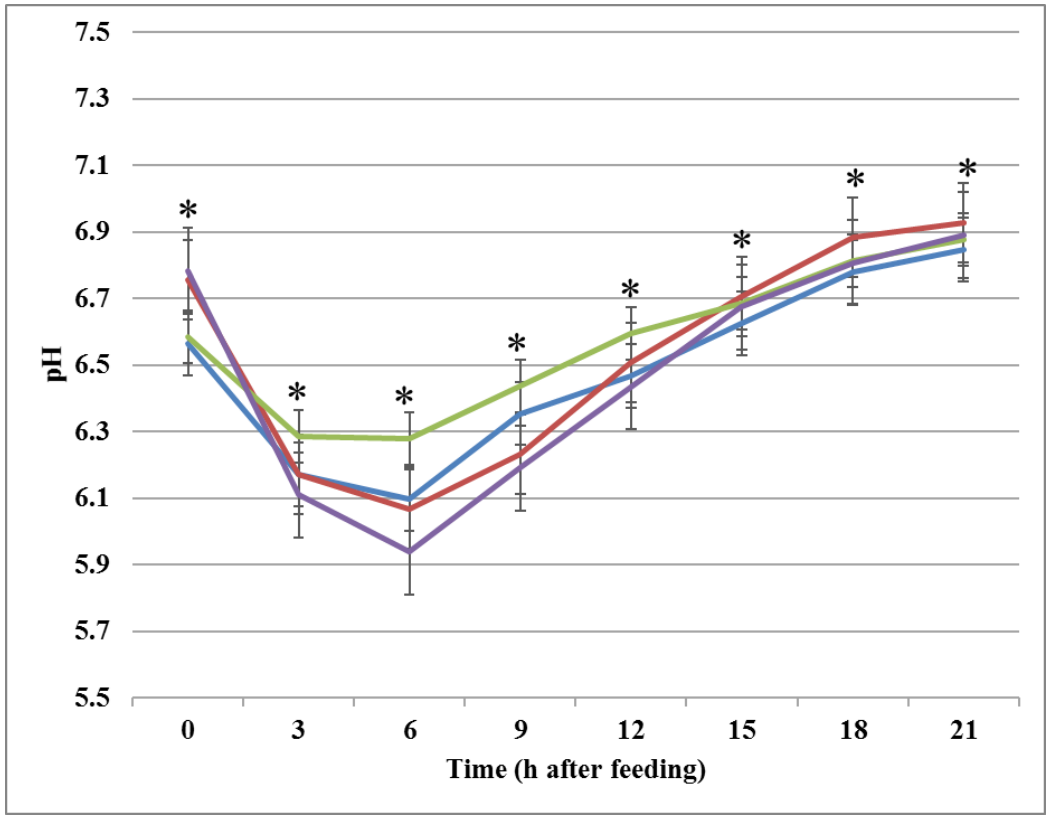


Figure 18 Ventilation design of enteric fermentation chamber to measure gas concentrations.



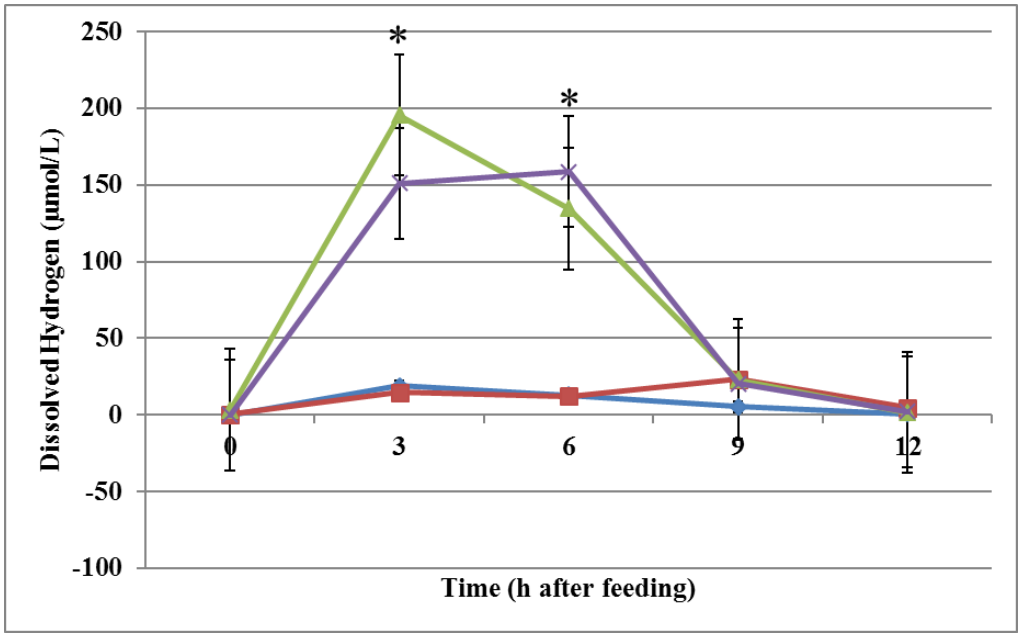
(McGinn et al., 2004).

Figure 19 Diurnal pH in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments over 24 h after feeding.



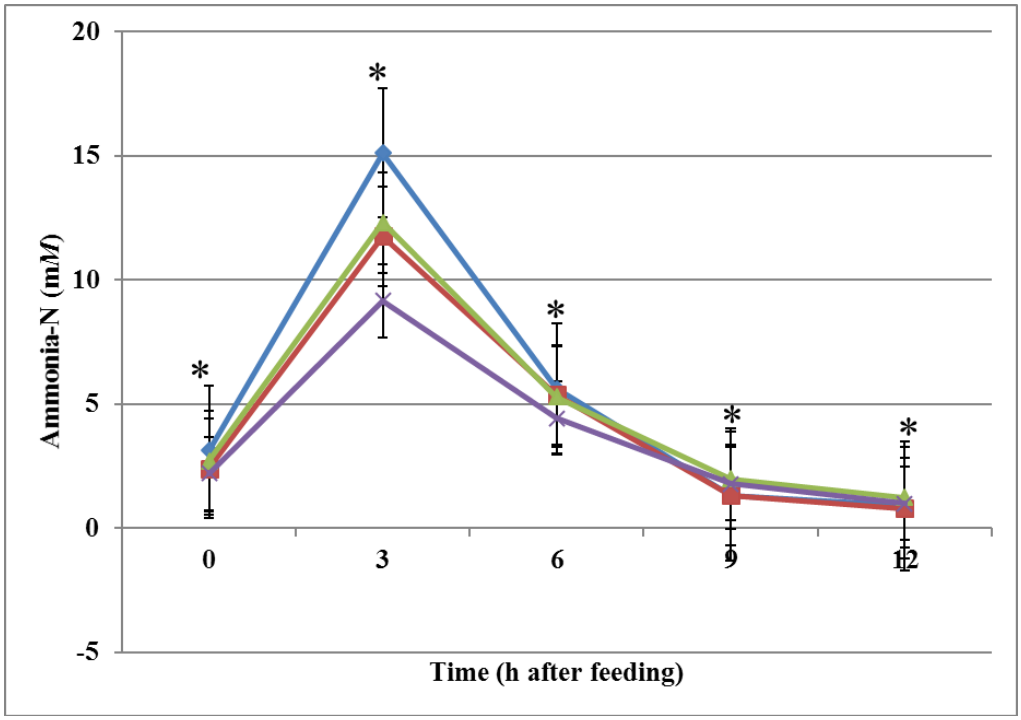
= CON (control), = OIL (canola oil), = NOP (3-nitrooxypropanol), and = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 20 Dissolved hydrogen concentration in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.



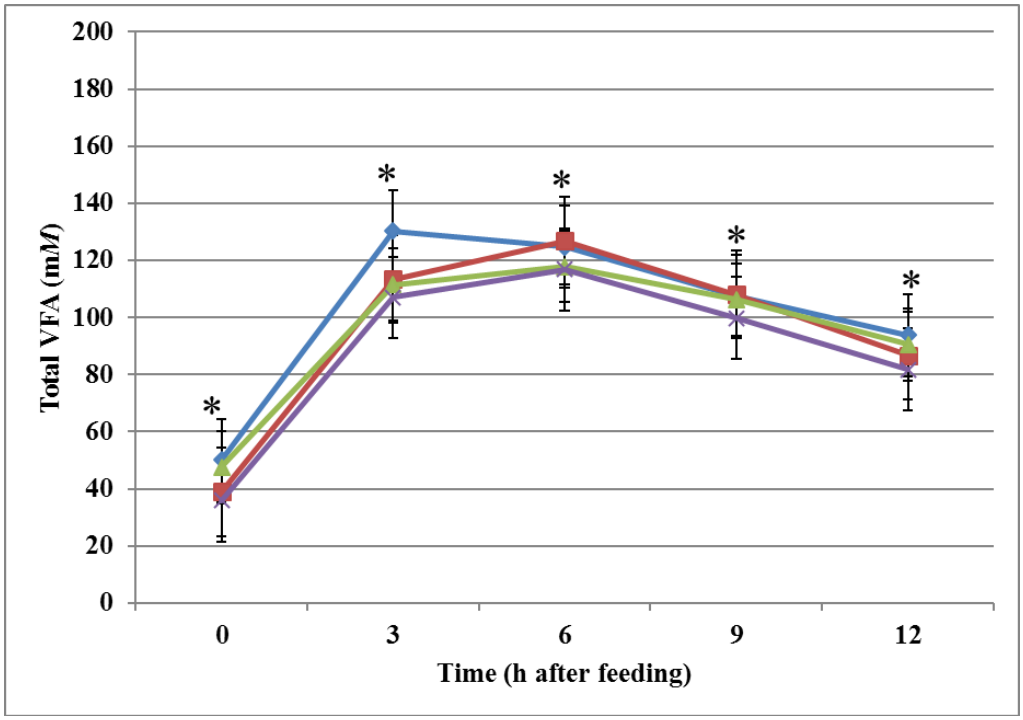
◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 21 Ammonia-N concentration in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

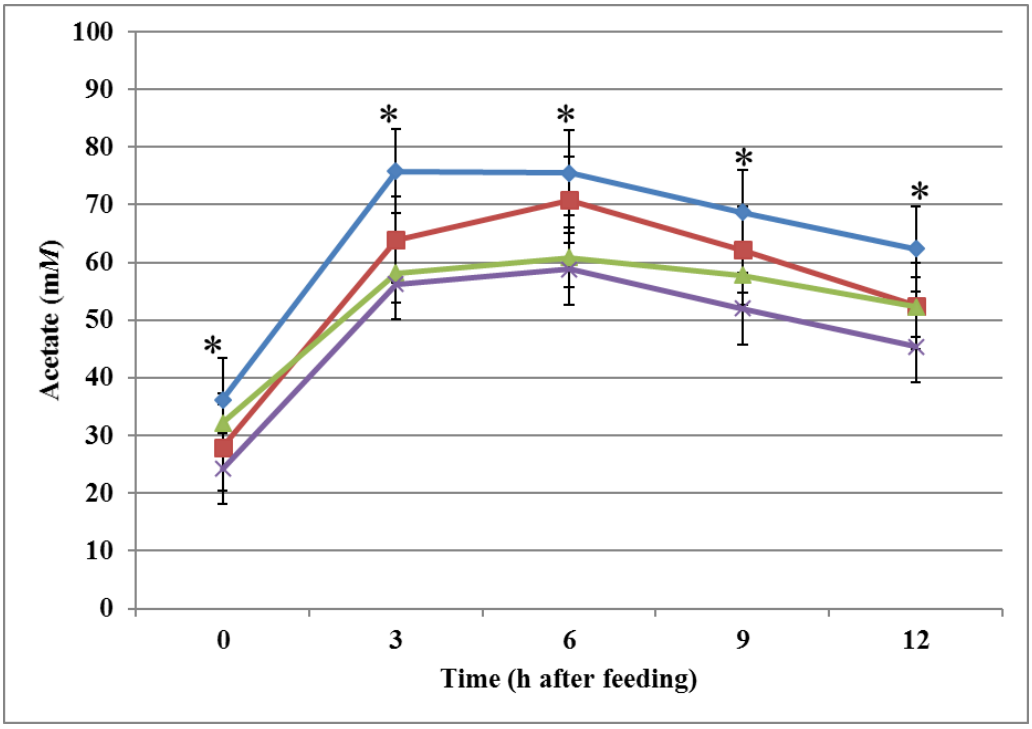
Figure 22 Total volatile fatty acid concentration in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 23 Acetate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

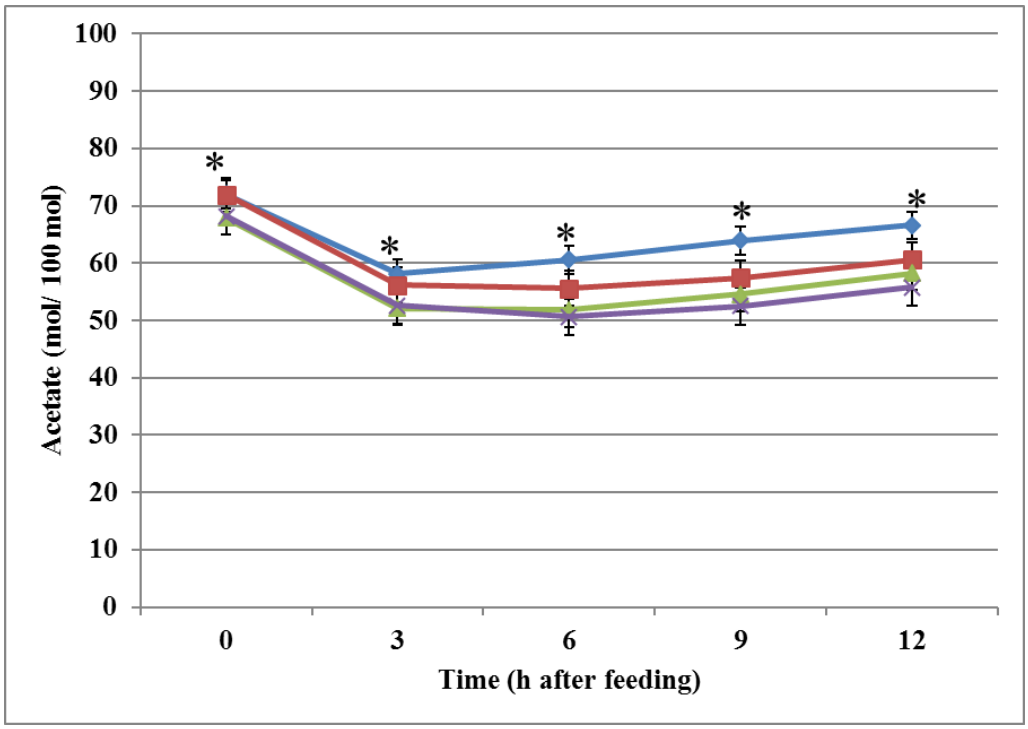
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Acetate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

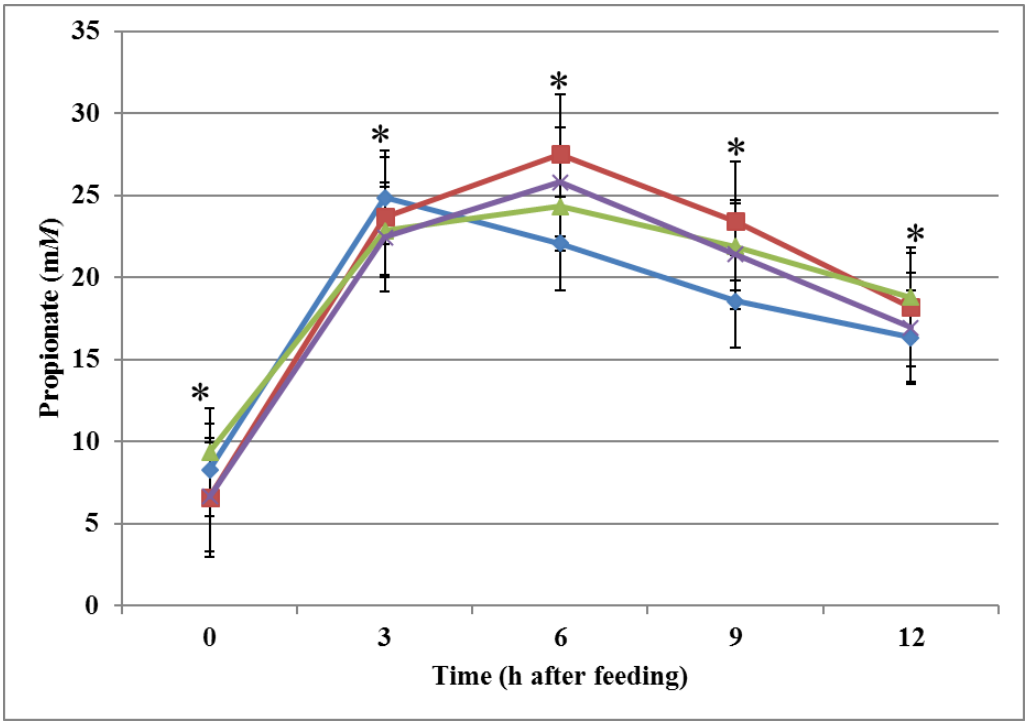
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 24 Propionate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

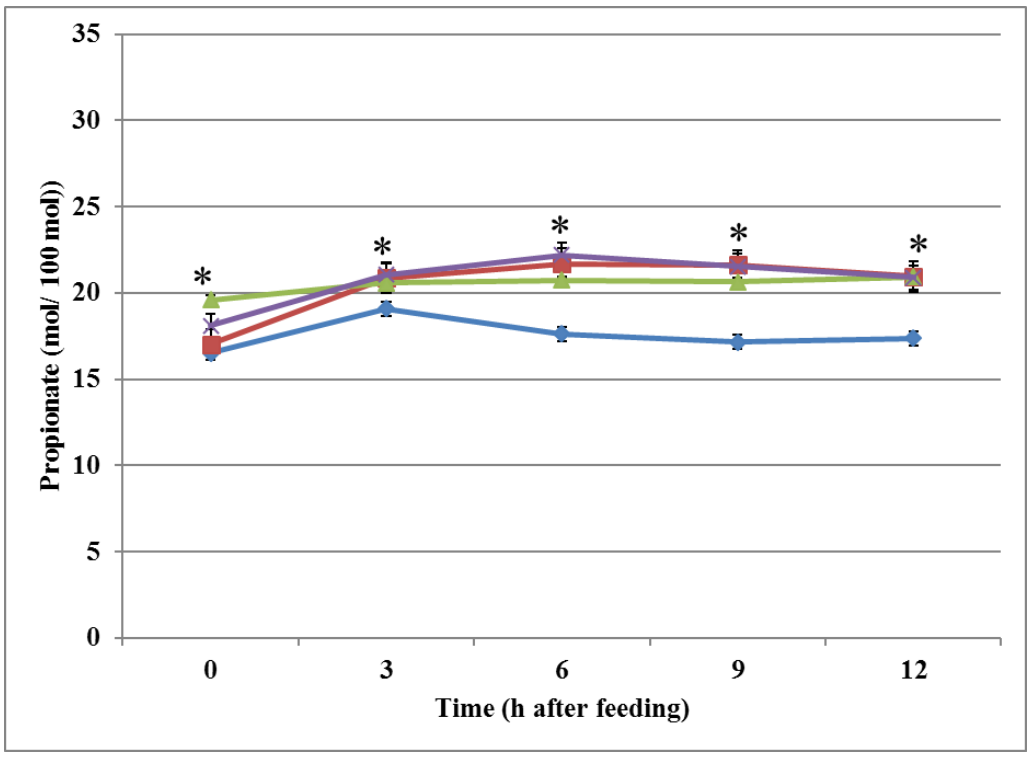
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Propionate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

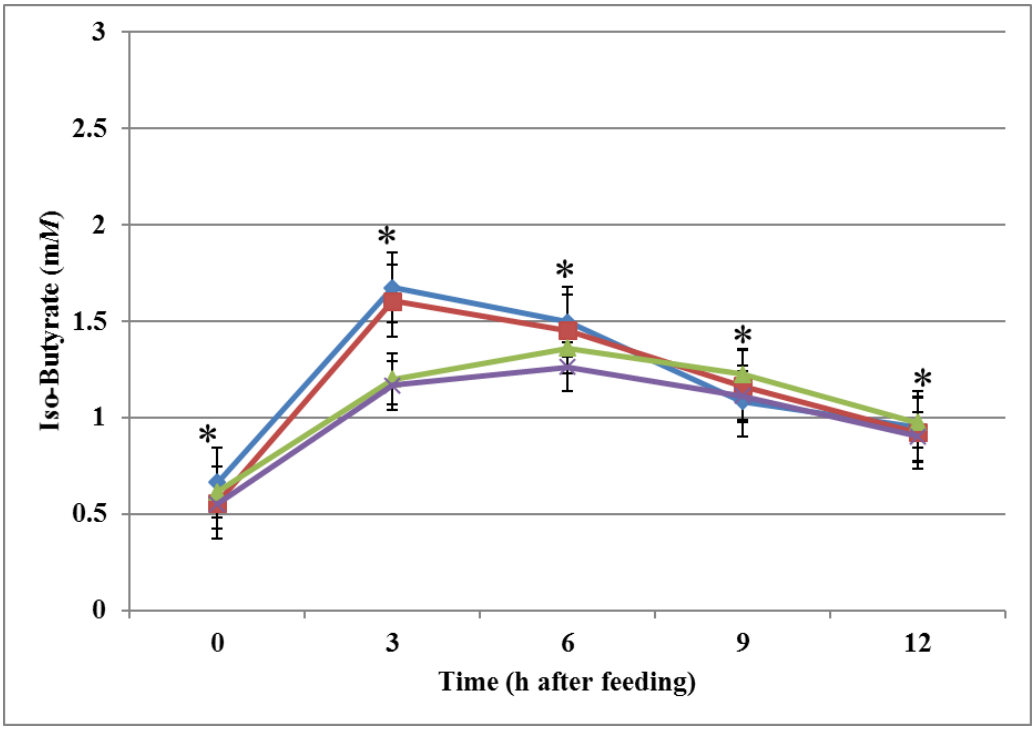
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 25 Iso-Butyrate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

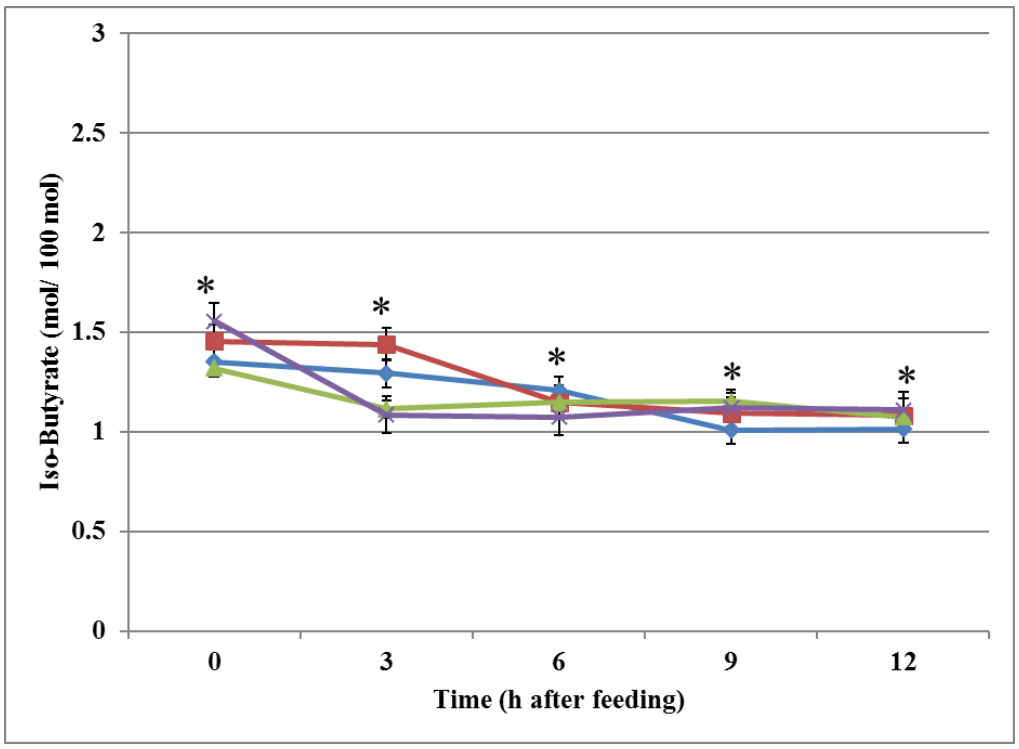
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Iso-Butyrate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

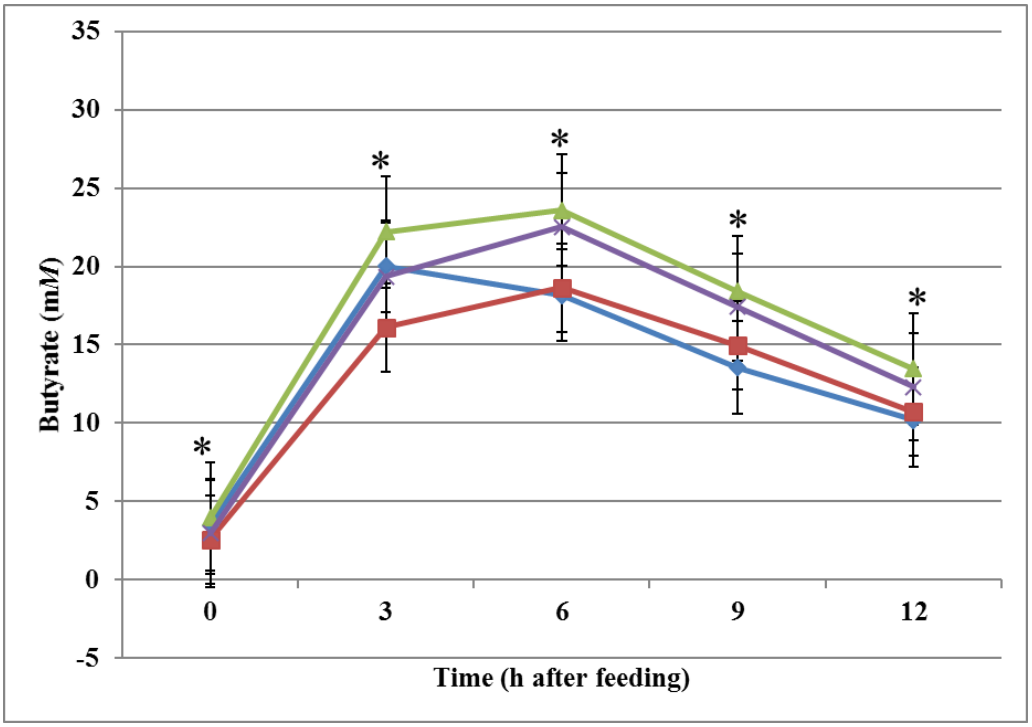
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 26 Butyrate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

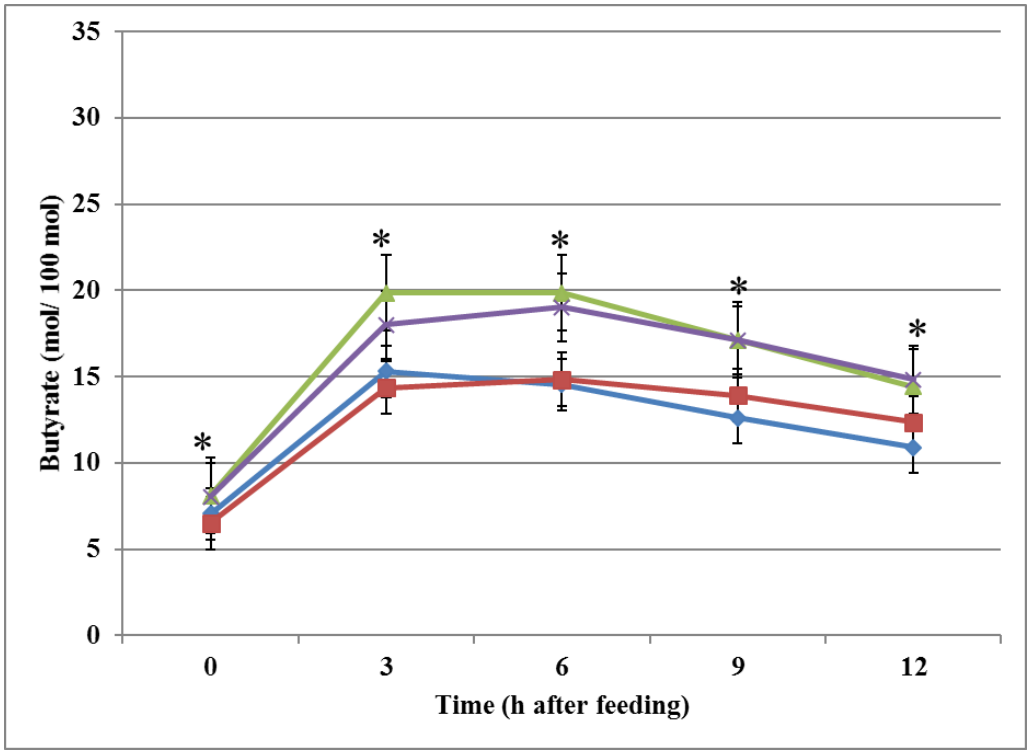
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Butyrate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

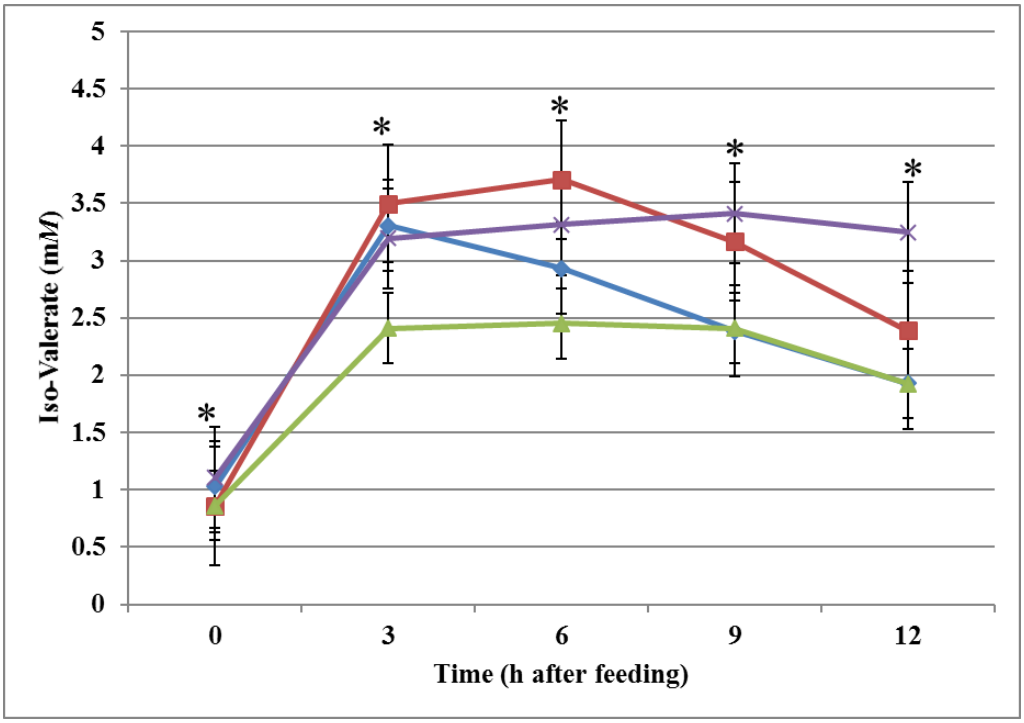
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 27 Iso-Valerate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

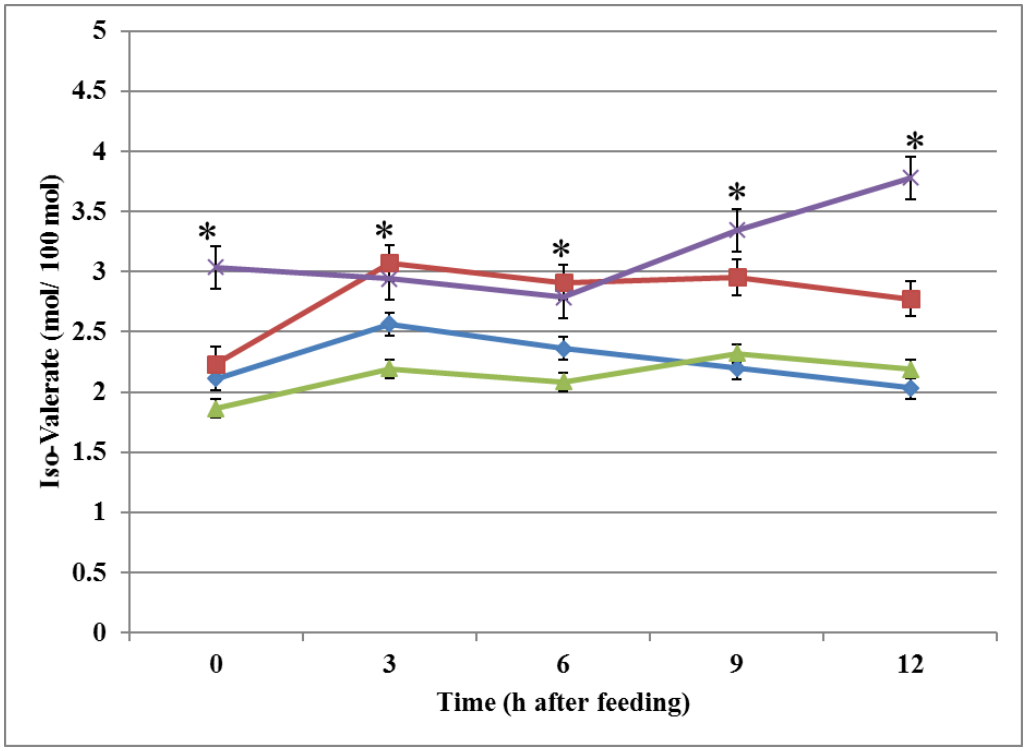
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Iso-Valerate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

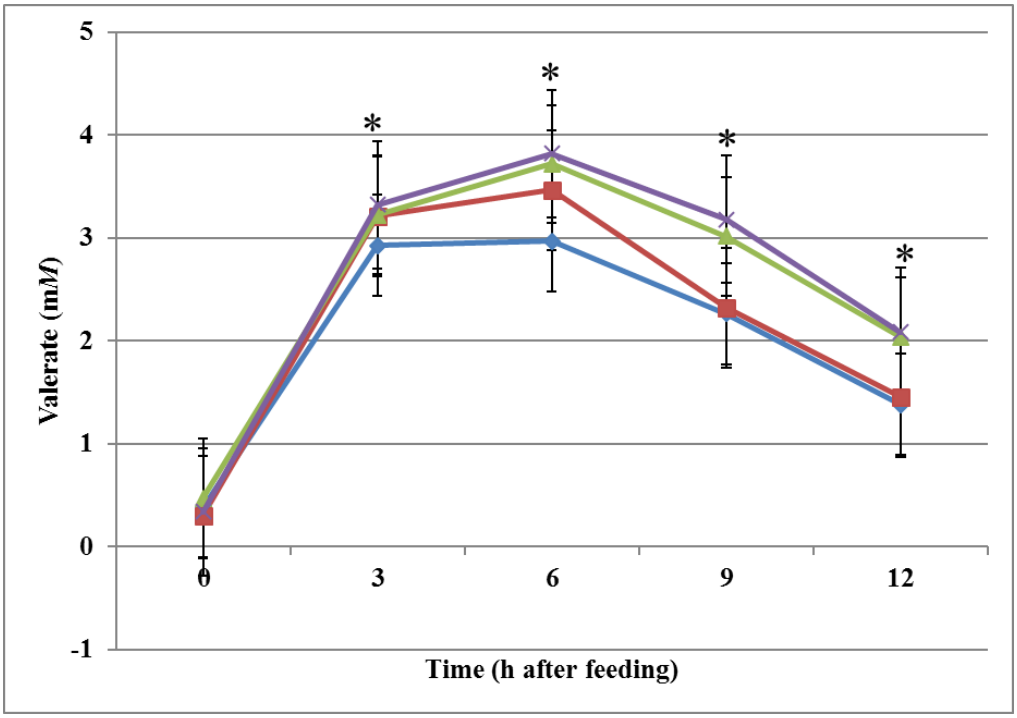
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 28 Valerate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

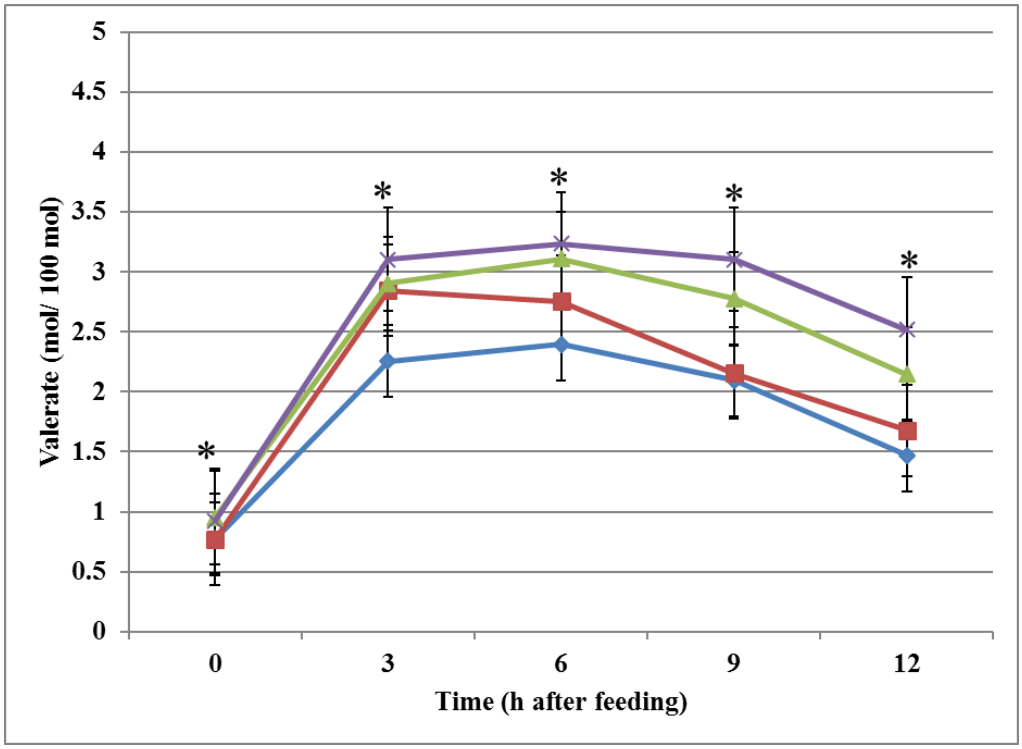
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Valerate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

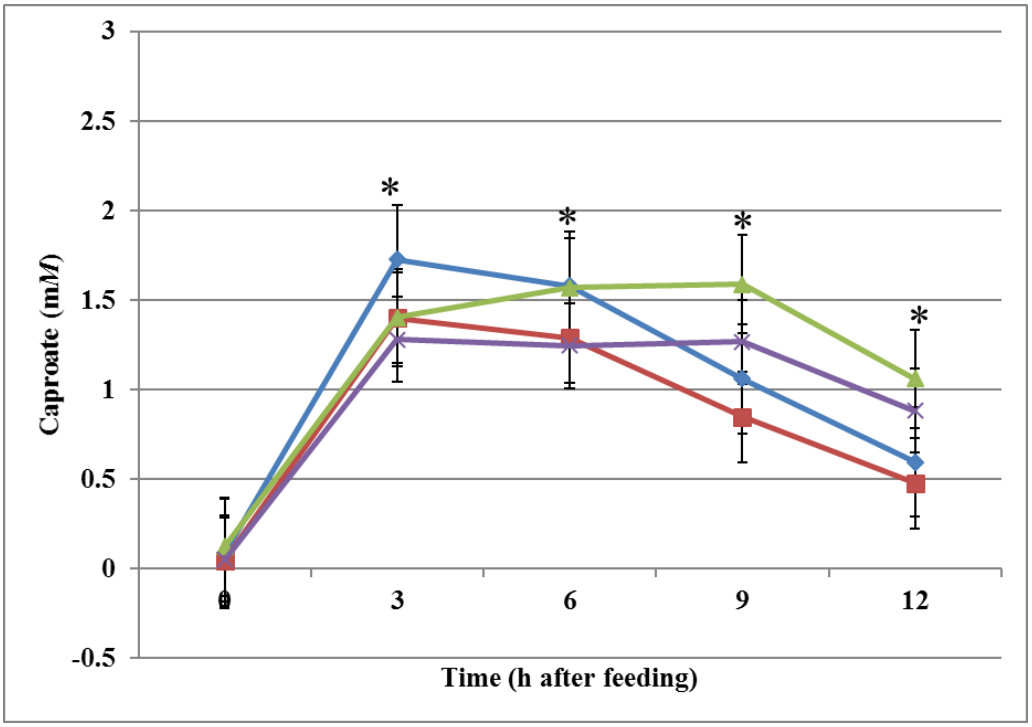
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 29 Caproate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

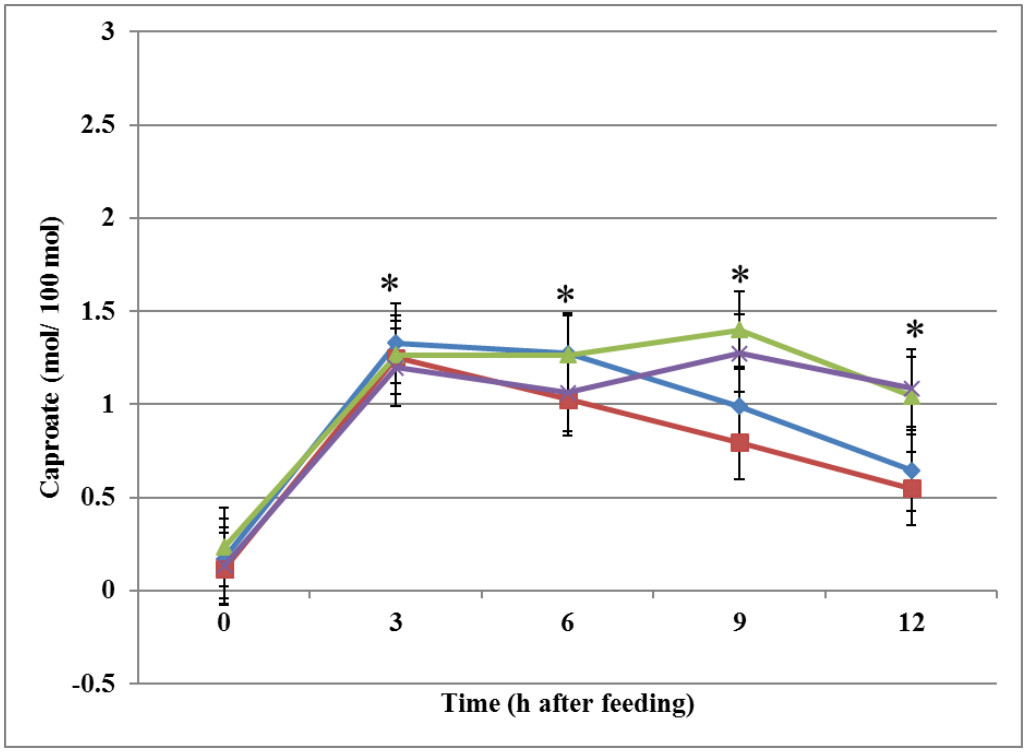
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Caproate concentration (a) and proportion (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

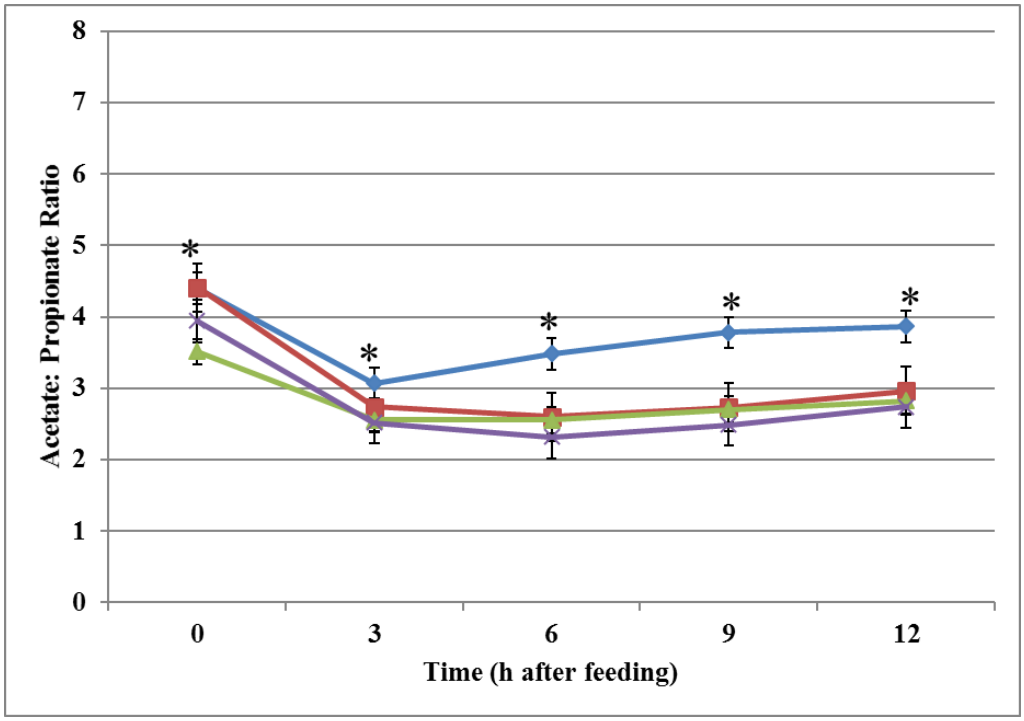
b)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 30 Acetate: Propionate ratio (a) and Acetate + Butyrate: Propionate (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding.

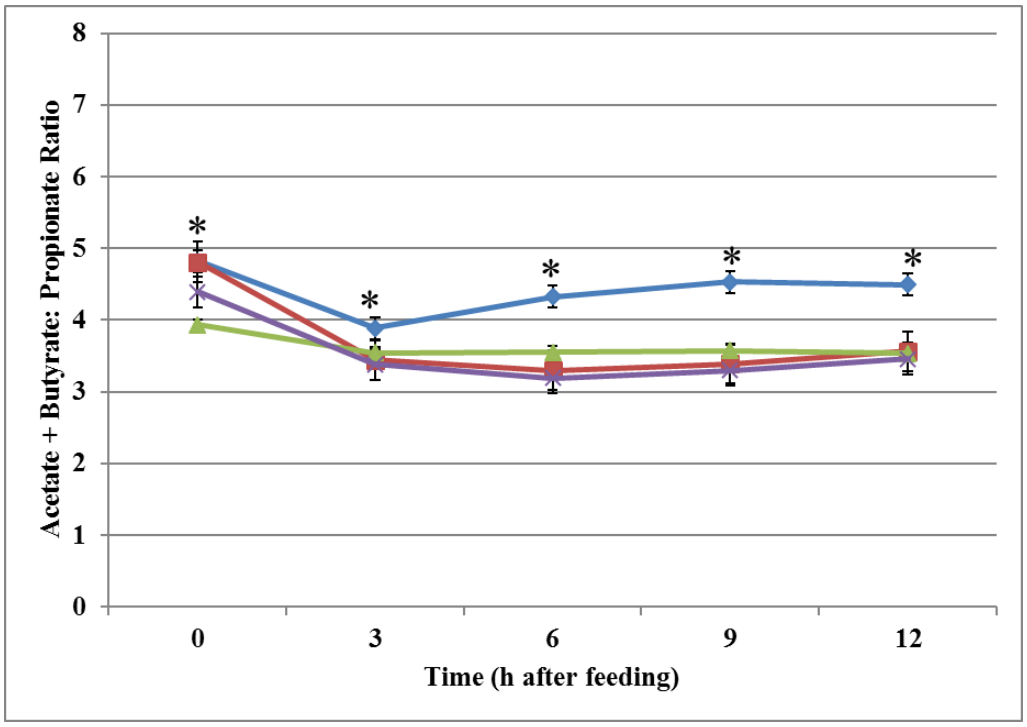
a)



◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and × = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

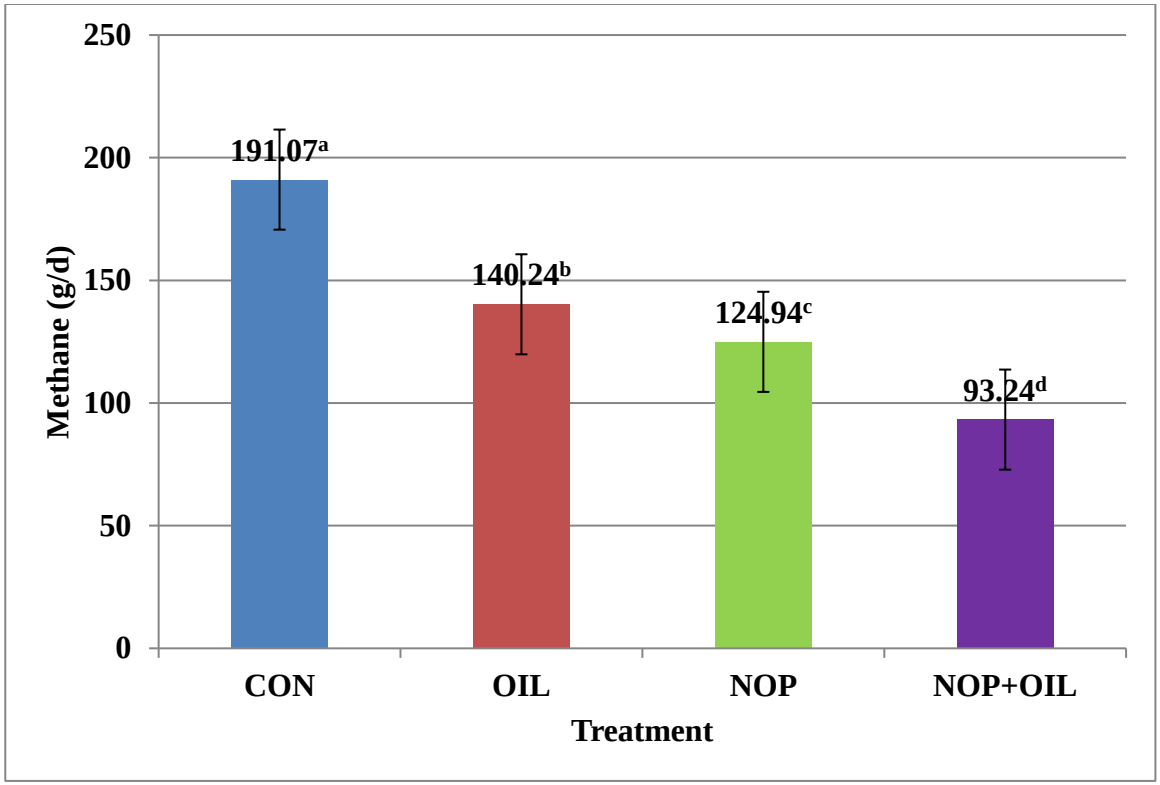
Acetate: Propionate ratio (a) and Acetate + Butyrate: Propionate (b) in rumen fluid of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments at 0, 3, 6, 9, and 12 h after feeding (continued).

b)



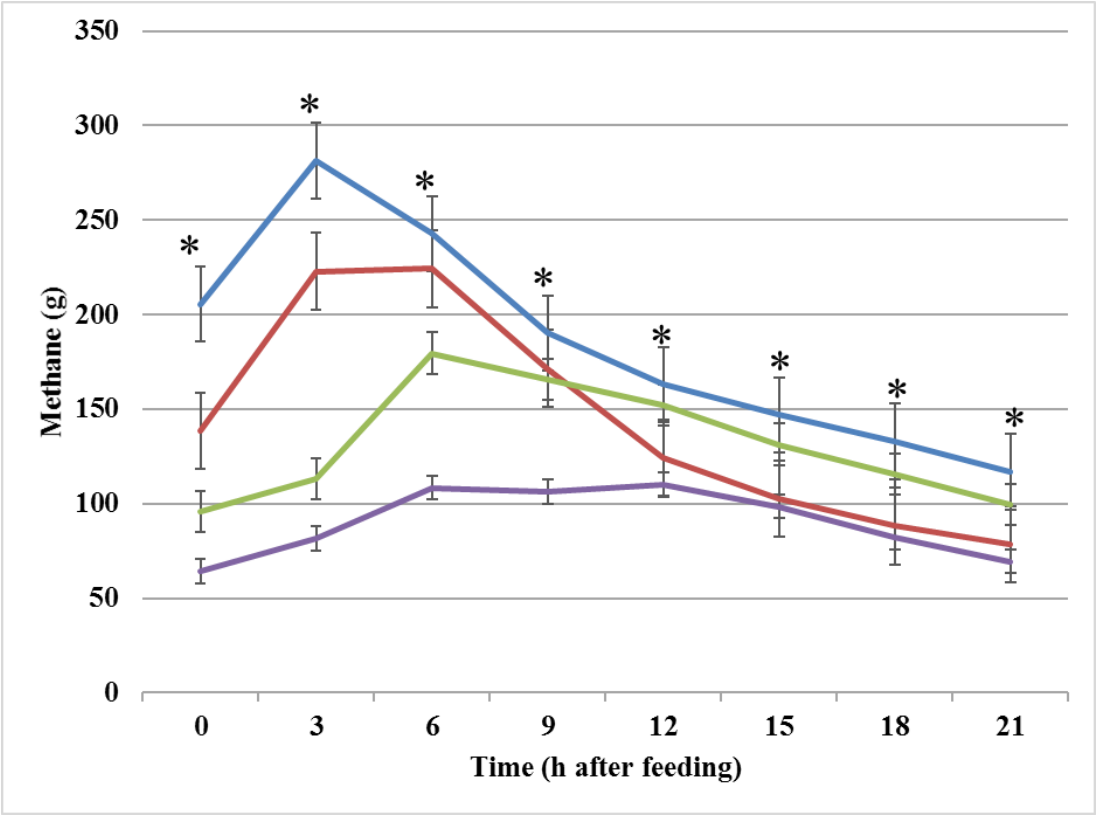
◆ = CON (control), ■ = OIL (canola oil), ▲ = NOP (3-nitrooxypropanol), and ✕ = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 31 Daily enteric methane production of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.



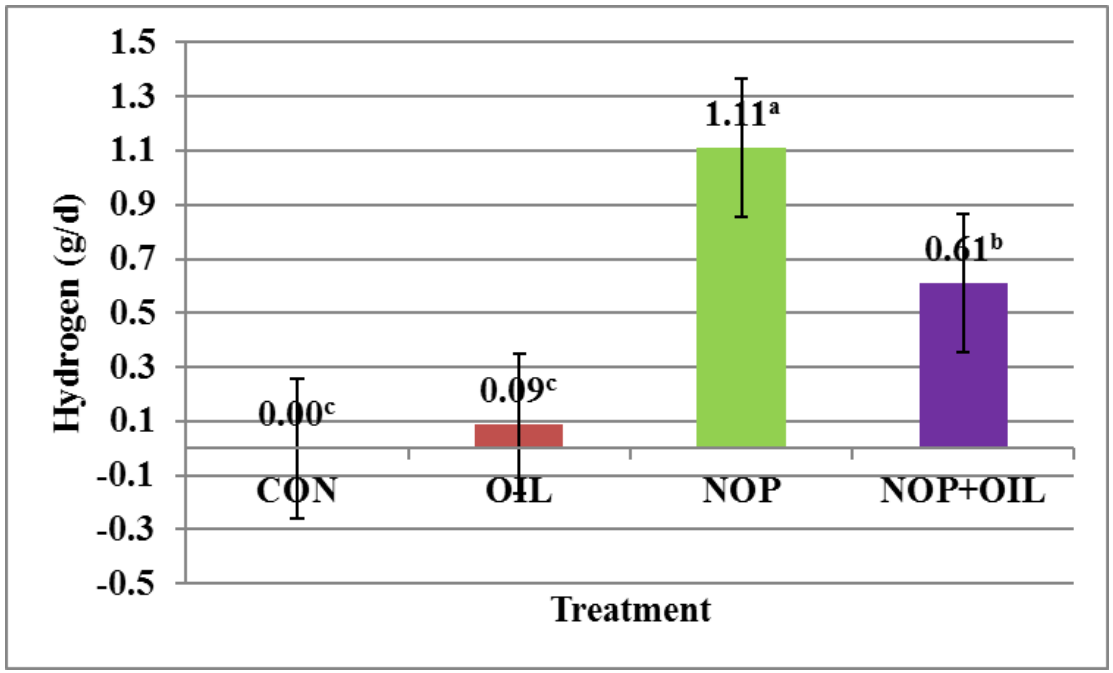
a-dLeast squares means with different superscripts differ (P < 0.05).
Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
Error bars indicate the SEM.

Figure 32 Enteric methane production of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments over 24 h after feeding.



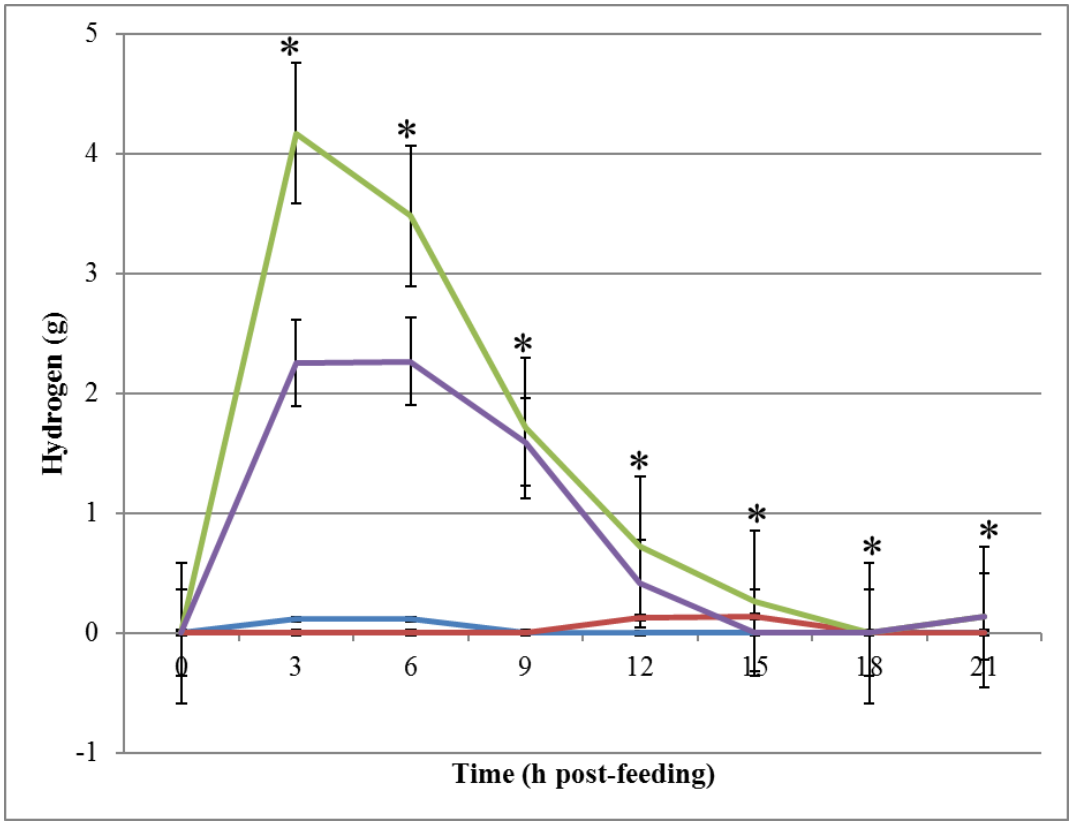
= CON (control), = OIL (canola oil), = NOP (3-nitrooxypropanol), and = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 33 Daily enteric hydrogen production of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments.



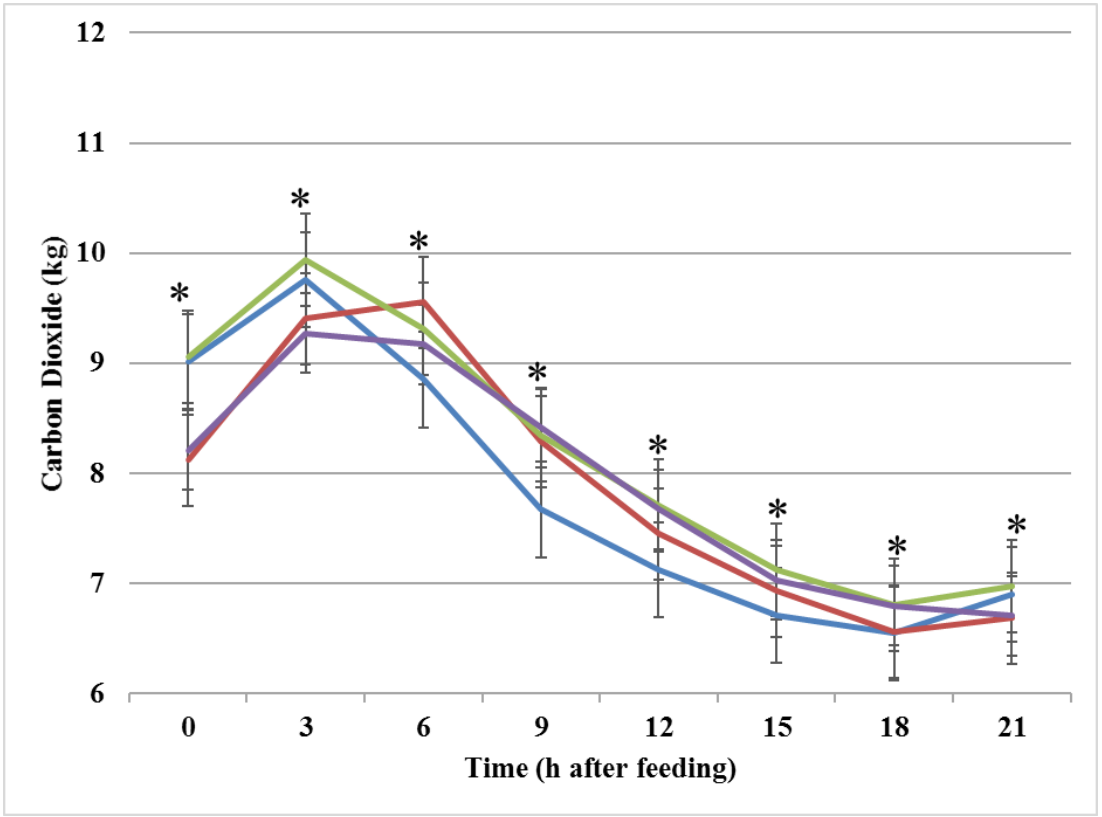
[a-c]Least squares means within a row with different superscripts differ ($P < 0.05$).
Treatments: CON= control, OIL= canola oil, NOP= 3-nitrooxypropanol, NOP+OIL= 3-nitrooxypropanol and canola oil.
Error bars indicate the SEM.

Figure 34 Enteric hydrogen production of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments over 24 h after feeding.



= CON (control), = OIL (canola oil), = NOP (3-nitrooxypropanol), and = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

Figure 35 Enteric and respiratory CO_2 production of animals fed the CON, OIL, NOP and NOP+OIL dietary treatments over 24 h after feeding.



= CON (control), = OIL (canola oil), = NOP (3-nitrooxypropanol), and = NOP+OIL (3-nitrooxypropanol and canola oil). Error bars indicate the SEM. Asterisks (*) indicate time points in which the main effect of treatment is significant ($P < 0.05$).

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Appendix

ANIMAL CARE COMMITTEE PROTOCOL AND APPROVAL

ANIMAL-USE PROTOCOL FORM

LETHBRIDGE RESEARCH CENTRE (LRC)
ANIMAL CARE COMMITTEE (ACC)

A minimum of 3 weeks turn around time should be planned for. Note that incomplete forms will be returned to the submitter. ALL white fields except Protocol # and signature lines should be filled in.

For more information, set to show comments by clicking on the "Review" tab, then "Show Markup" in the Tracking Section. Toggle comments on/off.

Protocol Title	**Assessing the potential of a novel feed additive and an unsaturated fat (canola oil) alone and in combination to lower methane emission from beef cattle and reduce their contribution to climate change.**
Funding Code (for tracking only):	
ACC Protocol #:	**1607**
Descriptive Keywords:	**Cattle, feed additive, methane, unsaturated fat**

Principal Investigator(s):	**Phone No(s):**	
Karen Beauchemin	317-2235	
Designated Emergency Contact(s):	**Phone No(s):**	**After Hours No(s):**
Karen Andrews **Bev Farr**	327-4561 - Ext: 3330 327-4561 - Ext: 3485	
Technical and other Authorized Personnel (list all):	**Phone No(s):**	**Most Recent Animal Use Training? (Title and Year)**
Karen Beauchemin **Karen Andrews** **Bev Farr** **Diwakar Vyas** **Nicholaus Johnson (co-op student)**		Animal Care 2015 Animal Care 2011 Animal Care 2012 Animal Care 2012 Animal Care 2015

Megan Smith (PhD student)		Dairy Animal Care and Quality Assurance (DACQA) Certification Program, Beef Check-off Master of Beef Advocacy (M.B.A.) Program, and Collaborative Institutional Training Initiative (CITI) Animal Care and Use Program.
Project Status	**If revision or renewal, give previous ACC Protocol #**	
[x] new [] revision []renewal		
Funding Approval	**[x] yes [] no [] pending**	
Funding Agency(ies)	**Climate Change and Emissions Management Corporation (CCEMC)**	
Start Date	**January 1, 2016**	
End Date (if this changes, you must contact the ACC)	**July 31, 2016**	

Issues covered by other Committees				**Committee Signature**
Biosafety concerns?	**[] yes [x] no**	**LBBC Approval**	**[] yes [] no**	
Radioactive material used?	**[] yes [x] no**	**RSO Approval**	**[] yes [] no**	
Feed Additives used?	**[x] yes [] no**	**FAAC Approval**	**[] yes [] no**	

Invasiveness category:	**[] A [x] B [] C [] D [] E**
Purpose of Animal Use:	**[] 0 [x] 1 [] 2 [] 3 [] 4 [] 5**
Anesthesia/analgesia?	No.
Euthanasia required?	No.
Animal or Carcass Disposition at end of Study	Following completion of this study, the animals will be retained in the herd for herd for use on subsequent studies or for a minimum of 4 weeks prior to slaughter. In the unlikely event that an animal needs to be sent to the abattoir before the end of the withdrawal period, the carcass will be rendered so it does

	not enter the food chain.
Veterinarian	Coaldale Vet Clinic 141 Broxburn Blvd (mail: Box 848, Coaldale, AB T1M 1M7) Lethbridge AB T1J 4P4 403-328-4454

Hazards to human health?	There are potential hazards during handling of large animals in the metabolism facility. Risks will be minimized by following the handling recommendations based on the institutional animal care training and wearing personal protective equipment according to Occupational Health and Safety guidelines.

Brief statement of objectives and procedures in lay terminology.	The objective of this study is to provide proof of concept that a novel feed additive (3-nitroxypropanol) and an unsaturated fat (canola oil) alone and in combination can mitigate methane gas emissions from cattle. Additionally, the effects of this additive and unsaturated fat on related parameters including diet digestibility, rumen fermentation, microbiome characterization, and abundance of MCRA genes will be observed.
Detailed Descriptions: *Objective(s) :* *Experimental Design and Statistical Analysis.* *Procedures:*	Background: AAFC-Lethbridge has been involved with DSM Nutritional Products (Switzerland) in the development and evaluation of a novel feed additive 3-nitrooxypropanol (NOP). Dietary supplementation of (NOP) has been shown to be a promising strategy because of its methane mitigation potential and lack of effects on diet digestibility. 3-Nitrooxypropanol is a structural analogue of methyl coenzyme M acting as an inhibitor of the enzyme methyl coenzyme M reductase during the last step of methanogenesis (Duval and Kindermann, 2012). It was shown that supplementing the diet of beef cattle (Romero-Perez et al., 2014; Romero-Perez et al., 2015), dairy cows (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015), and sheep (Martinez-Fernandez et al., 2014) with NOP resulted in significant reduction in enteric methane production. Moreover, NOP was shown to have no negative effects on feed intake or digestibility (Romero-Perez et al., 2014) and posed minimal risk in terms of food safety. Because methane emissions account for up to 12% of gross energy intake of cattle, it is possible that reduced methane emissions might spare metabolizable energy that could be utilized by the animal to meet its requirements for growth. In order to register this compound in Canada, further evaluation in cattle is necessary. Our team has been searching for new ways to reduce enteric methane in cattle for the past decade – this goal has proven to be very difficult and to date, we

	have not found an additive that reduces emissions in vivo to a greater extent that NOP. In addition, inclusion of feed ingredients high in unsaturated fatty acids has been demonstrated to reduce enteric methane emissions in vivo (Beauchemin et al., 2009 and Shinkai et al., 2012), and this practice is the generally accepted method of reducing methane in commercial beef and dairy operations. The potential interaction between these two approaches to mitigating methane emissions (NOP and fat) has not been examined. If NOP, alone or in combination with an unsaturated fat, reduces emissions in cattle, the benefit to Canadian farmers would be substantial. Agriculture accounts for 9% of the country's greenhouse gas emissions, and half of those emissions are from methane (90% of from enteric methane). Therefore, a 30% reduction in methane from livestock would be significant. Having our team at the forefront of this initiative will allow us to confirm/refute the claim for this additive. It will also allow us to incorporate these results in our greenhouse gas modeling and life cycle assessment of beef and dairy products. Objectives: The primary objective of this study is to determine whether the effects of combined methane mitigation strategies, inhibitor (NOP) and unsaturated fat, are additive, synergistic, or antagonistic, on methane gas emissions from cattle. Additionally, the effects of these additives on related parameters including intake, diet digestibility, rumen fermentation, microbial populations and abundance of MCRA genes will also be investigated. Experimental design: Eight, previously ruminally cannulated cattle will be used in this study to examine the effects of dietary supplementation with a novel feed additive, alone and in conjunction with an unsaturated fat, on methane emissions and related parameters. This experiment will be set up a double 4 x 4 Latin square design with four 28-d periods and four dietary treatments. The diet treatments are: 1) control (no NOP, no fat), 2) NOP alone, 3) unsaturated fat (canola oil) alone, and 4) NOP and fat combined. The dose of NOP will be 200 mg/kg of diet based on previous studies. Unsaturated fat (canola oil) will be added at 5% of the diet dry matter. Each treatment will be offered by mixing it with the basal diet. The diet will be fed once daily as a total mixed ration, and will contain: Barley silage (60%), barley grain (35%), and mineral/vitamin supplement (5%). All diets will be formulated to meet the nutrient requirements according to the NRC (1996). The animals will be adapted to the supplemented diets by stepping up the amount fed gradually over a 7-d period. Upon stepping up the supplemented diets, if dry matter intake on a particular day drops by more than 20% (based on the 3 previous days), the supplementation rate will not be further increased until intake rebounds. If it does not rebound, the supplementation rate will be decreased.

The two groups of animals will be staggered by 1-2 weeks, to make sampling easier. Animals will be housed separately (individual stalls) for the duration of the study. Animals will be exercised daily (except when in the chambers).

Statistical analysis: Normality of distribution and homogeneity of variance will be determined using the Univariate procedure of SAS (SAS Inst., Inc., Cary, NC). The data will be subsequently analyzed using a mixed procedure of SAS. For all traits of interest the animal will be the experimental unit. For methane emissions and diet digestibility, the statistical model will include block, treatment, period and the random effect of animal. Rumen fermentation data will be analyzed as a repeated measure including the fixed effects as previously described and including sampling time, with the random effect of animal.

Procedures:
Adaptation phase: Day 1-14 of each experimental period will be adaptation phase for animals to adapt to various treatments.

Rumen fermentation: To determine the treatment effects on rumen pH, diurnal pH profiles will be measured starting at feeding on day 15 and remain in the rumen for 7 days (which includes the period of methane measurement) using an indwelling pH data acquisition system (LRCpH dataloggers). The pH will be recorded every minute. The systems will be calibrated (in pH4 and pH7 solutions) prior to insertion on the first day and then upon removal on the last day (d-22). On day 15, samples of rumen fluid and solids (1 litre of contents/animal/sampling) will be obtained prior to feeding and at 3 h intervals post feeding for a period of 12 h (0,3,6,9,12h) for VFA, ammonia, protozoal and microbial analysis, dissolved hydrogen, biohydrogenation intermediates, and abundance of Methyl Coenzyme M Reductase A (MCRA) genes.

Methane measurements: The methane measurements will be carried out using the environmental chamber technique from d 19-22. All animals will be trained for entry into the chambers prior to starting the trial. For these measurements, one animal will be assigned (according to block) to each of the environmental chambers for a period of three days. Animals will be closely monitored and will be removed from the chamber should they exhibit signs of distress. Each of the four chambers (4.4 m wide x 3.7 m deep x 3.9m tall) will house one animal in a stall. The total quantity of methane emitted in the chambers will be is quantified by measuring the gradient of influx and exhausted concentrations and volumes. The air volume in each chamber will be exchanged every 5 min. Methane concentration will be recorded every 30 minutes by a calibrated infrared gas analyzer.

	Diet digestibility: Following methane measurement, animals will be given a rest period of 2d (d-22-24) prior to the commencement of diet digestibility determination. Feed intake will be restricted to 0.95 of ad libitum consumption (determined from the day 10 to 17 intake, prior to the animals going into the chambers) starting at feeding on Day 25 (Mon). Starting on day 25 (Mon), animals will be housed in the metabolic stalls for four days (d 25-28). The total fecal collection technique will be employed for this analysis, with the total weight of feed, orts, feces and urine recorded daily. Urine will be collected via the installation of urinary catheters. Each day, 10 % sub samples will be collected from feed, orts, and feces and pooled at the end of each period on an individual animal basis. These samples will be stored for subsequent DM determination and chemical analysis.
References:	Beauchemin, K. A., S. M. McGinn, C. Benchaar, and L. Holtshausen. 2009. Crushed sunflower, flax, or canola seed in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. J. Dairy Sci. 92: 2118-2127. Duval, S., and M. Kindermann. 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 84629: A1. Haisan, J.,Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97: 3110-3119. Hristov, A. N., O. Joonpyo, G. Fabio, Y. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moate, M. H. Deighton, S. R. O. Williams, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. 112: 10663-10668. Martinez-Fernandez, G., L. Abecia, A. Arco, G. Cantalapiedra-Hijar, A. I. Martín-García, E. Molina-Alcaide, M. Kindermann, S. Duval, and D. R. Yáñez-Ruiz. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. J. Dairy Sci. 97: 3790-3799. Reynolds, C. K., D. J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 97: 3777-3789.

	Romero-Perez, A. E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682-4693. Romero-Perez, A. E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2015. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. J. Anim. Sci. 93: 1780-1791. Shinkai, T., O. Enishi, M. Mitsumori, K. Higuchi, Y. Kobayashi, A. Takenaka, K. Nagashima, M. Mochizuki, and Y. Kobayashi. 2012. Mitigation of methane production from cattle by feeding cashew nut oil. J. Dairy Sci. 95: 5308-5316.
Experimental Endpoint(s):	Following completion of this study, the animals will be retained in the herd for use on subsequent studies or for a minimum of 4 weeks prior to slaughter. In the unlikely event that an animal needs to be sent to the abattoir before the end of the withdrawal period, the carcass will be rendered so it does not enter the food chain.
Humane Intervention:	Animals will be monitored daily for any signs of illness or stress. Sick or injured animals will be removed from the group for treatment after consultation with a veterinarian (Coaldale Veterinary Clinic).
Animal Use Considerations	
Replacement of Animals	Methane emission from ruminants is the result of a complex series of digestive and absorptive processes that occur in the intestinal tract, many of which cannot be simulated using in vitro methodologies. Thus, in vivo measurements are warranted in this study.
Reduction of Number Used	The number of animals used in this study cannot be further reduced in order to ensure adequate replication for statistical comparisons (n=8/treatment).
Refinement of Procedures	All procedures have been used in previous published studies that have been used previously in our lab and have been subjected to peer review previously.
Animals	
Species (Breed or Strain)	Beef heifers
# Requested	8 (previously cannulated)

Location/Facility	Adaptation & rumen sampling: Metabolism barn Methane measurement: Controlled environment building Diet digestibility measurement: Metabolism barn
Housing/Husbandry	Standard husbandry practices will be employed in this study. During adaptation, rumen sampling and methane measurement animals will be fed once daily for ad libitum intake. During diet digestibility determination, intake will be restricted to 95% of ad libitum based on intake recorded over the five days prior to sampling commencement.
Detailed Justification of Number of Animals and Species Requested: *CCAC requires some consultation with a statistician to determine required animal numbers. To determine power and sample size calculation go to:* http://homepage.stat.uiowa.edu/~rlenth/Power/index.html *Choose the appropriate method and calculate power. Report the value for animal number justification. If you have any difficulties in determining animal numbers, consult the statistician directly. Please indicate any further details justifying the number of animals requested.*	
A minimum of 8 animals is required for meaningful comparisons between treatments (n=8). Reducing the number of animals further would prevent the statistical requirements for this type of experimental design being met.	

ACC (comments, concerns, discussions):

1. When cattle are in the environmental chambers, what would be tolerated distress behaviour? What signs of distress would have to be displayed to be removed from the chamber? How often has this happened in past research trials?

   Figure 1 The major sign of distress would be a drastic reduction in feed intake. If an animal is stressed because of a new environment it will refuse to eat or drink, and sometimes it will refuse to lie down and ruminate. We have been using the environmental chambers since 2001, and over that time we have learned how to minimize stress on the animals. Firstly the animals are adapted to the chambers before starting the study. They are "halter broken" meaning they are trained so they are used to standing in a stall. Next, they spend time in the chambers over night before starting the study so they know what to expect. Mirrors are placed in the chambers so the animal perceives that it is not alone. We also provide them with "toys" such as balls that they can play with. Occasionally we have an animal that doesn't eat, or eats minimally. We give it 24 h, and if the situation doesn't improve we remove the animal. That has happened a couple of times in the past 15 years (2x? or 3x ...not sure).

   Figure 2

2. If an animal is injured or gets sick will it be replaced on the trial?

   Yes, another animal will be used from the pool of cannulated animals.

LRC ANIMAL CARE COMMITTEE

December 2, 2015 **File: 100-1-22**

Memorandum To: **Dr. K. Beauchemin**
Project Leader

From: **(vacant)**
Chairperson, Animal Care Committee

Subject: **Animal Use Protocol Review (1607)**

The Animal Care Committee (ACC) collectively reviewed your protocol entitled **"Assessing the potential of a novel feed additive and an unsaturated fat (canola oil) alone and in combination to lower methane emission from beef cattle and reduce their contribution to climate change."** at the last committee meeting on December 1, 2015.

The committee has granted this protocol full approval as written.

During any future correspondence with the ACC please ensure to quote Protocol #1607 for this project.

If you have any further questions please contact my office at 317-2248.

cc: D. Vyas
K. Andrews
B. Farr
G. Steacy

BP/