Effect of biochars differing in source, inclusion level and post-pyrolysis treatment on *in vitro* methane production and fermentation of a barley silage-based beef cattle diet

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THESIS ABSTRACT

This study comprised four *in vitro* experiments, two batch culture and two Rumen Simulation Technique (RUSITEC), to evaluate the potential of biochar to mitigate enteric methane (CH_4) in beef cattle diets. Biochar products used in the study were coconut (CP001 and CP014) or pine (CP002, CP015, CP016, CP023, CP024), differing in their physical and chemical composition. In the batch culture, they were evaluated at different levels of inclusion (Exp. 1: 4.5, 13.5 and 22.5 %; Exp. 2: 2.3 and 4.5% diet DM) and particle size (Exp. 2: < 0.5, 0.5-2.0, > 2.0 mm) to determine effects on DM disappearance (DMD), total gas and CH_4 production and ruminal fermentation parameters (pH, volatile fatty acids (VFA), and ammonia nitrogen (NH_3-N)) when added to a barley silage-based diet. In Exp. 1, level of biochar inclusion linearly ($P < 0.01$) decreased DMD but had no effect ($P > 0.05$) in Exp. 2. In both experiments, total gas production and CH_4 were not affected ($P > 0.05$) by biochar treatment nor level of inclusion. Rumen fermentation parameters were also not affected by treatment ($P > 0.05$) or level of inclusion ($P > 0.05$) in either experiment. Additionally, particle size had no effect on any measured parameters ($P > 0.05$). Subsequently, two RUSITEC experiments evaluated: 1) three pine-based biochars (CP016, CP023, CP028), and 2) three spruce-based biochars treated post-pyrolysis with salt ($ZnCl_2$) or acids (HCl/HNO_3 or H_2SO_4), respectively. In both experiments, biochar was included in a barley silage-based diet at 2 % of diet DM. Biochar did not affect ($P > 0.05$) nutrient disappearance parameters (DM, OM, CP, NDF, ADF or starch disappearance), total gas or CH_4 production in either experiment *(*$P > 0.05$). Rumen fermentation parameters ($P > 0.05$), total protozoa counts ($P > 0.05$) and microbial protein synthesis were also unaffected ($P > 0.05$). Lastly, alpha and beta diversity and rumen microbiota families were unaffected by biochar ($P > 0.05$), except for family Rikinellaceae. In conclusion, biochar did not offer the potential to mitigate enteric CH_4 emissions nor improve rumen fermentation parameters in a barley silage-based diet in either batch culture or RUSITEC.

Keywords: biochar, methane, nutrient digestibility, rumen fermentation, rumen microbiota, RUSITEC, total mixed ration

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DEDICATION

I dedicate this manuscript to my family as with their never ending support, patience and encouragement I was able to continue and push further through my endeavors.

FOREWORD

The chapters in this manuscript are formatted in accordance to the journal that they were or will be submitted to. The first chapter comprised two *in vitro* batch culture experiments and is formatted according to the Canadian Journal of Animal Science (CJAS) which is the target journal. The second and third chapters were RUSITEC experiments that are currently under review with CJAS and Animal Feed Science and Technology, respectively. Each chapter consisted of an abstract, introduction, materials and methods, results, discussion and conclusions. This thesis also includes a literature review, general discussion and conclusions.

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ABBREVIATIONS

ADF = acid detergent fiber

ADFD = ADF disappearance

BCVFA = branched chain VFA

°C =degrees celsius

C = carbon (element)

CFIA = Canadian Food Inspection Agency

CH_4 = methane

CO_2 = carbon dioxide

CP = crude protein

CPD = CP disappearance

DADA2 = divisive amplicon denoising algorithm

dL = deciliter

DM = dry matter

DMD = DM disappearance

DMI = DM intake

DNA = deoxyribonucleic acid

dsDNA = double-stranded DNA

Exp = experiment

FA = fatty acid

FPA = feed particle-associated bacteria

FPB = feed particle-bound bacteria

g = gram

GC = gas chromatography

GHG = greenhouse gas

H_2SO_4 = sulfuric acid

H= hydrogen

h = hour

HCl = hydrochloric acid

HNO_3 = nitric acid

IU = international unit

IVOMD = *In vitro* organic matter disappearance

L = liter

LAB = liquid-associated bacteria

LEfSe = linear discriminant analysis

mg = milligram

mm = millimeter

mmol = millimolar

ml = milliliter

mo = month

N_2O = nitrous oxide

N = nitrogen

NDF = neutral detergent fiber

NDFD = NDF disappearance

NH_3-N = ammonia nitrogen

OM = organic matter

OMD = OM disappearance

OTU = operational taxonomic units

P = phosphorous

PEAR = Paired End Re(A)d Merge(R)

PCR = polymerase chain reaction

PyNAST = python nearest alignment space termination

rRNA = ribosomal RNA

RUSITEC = rumen simulation technique

S= sulphur

SEM = standard error of the mean

TMR = total mixed ration

VFA = volatile fatty acid

$ZnCl_2$ = zinc chloride

GENERAL INTRODUCTION

Agricultural commodities are essential for global food security, with the demand for meat and livestock products expected to continually grow with the expanding human population (Rojas-Downing et al. 2017). Concurrently, climate change and global warming are garnering increased attention and scrutiny on farming, including animal husbandry, as these result in the production of greenhouse gases (GHG). The agricultural sector contributes approximately 26 % of global anthropogenic GHG (Turbiello et al. 2015; Frank et al. 2017), with 14.5 % of these emissions coming from the livestock industry and related anthropogenic practices (Gerber et al. 2013; Rojas-Downing et al. 2017). Of the agricultural GHGs, emissions from livestock production have been the subject of particular public consideration in Canada, as this sector accounts for 40% of total agricultural emissions (Environment Canada 2019). In ruminant livestock, enteric CH_4 is produced from digestive processes and accounts for 2-12 % of gross energy intake (GEI), with diet type and digestibility accounting for this variability (Johnson and Johnson 1995). Dietary manipulation is one of the most successful strategies that has been identified to mitigate CH_4 (Haque 2018), and includes the following strategies: increasing dietary fat content (Beauchemin et al. 2008; Martin et al. 2010); improving feed quality (Boadi and Wittenberg 2002; Degola et al. 2015); incorporating starch or grain-based feeds (Johnson and Johnson 1995; Mitsumori and Sun 2008); and the inclusion of novel feed additives and supplements (Knapp et al. 2014).

In the area of feed additives, there has been a growing interest in the use of biochar in cattle diets. However, this is a relatively novel dietary approach and its effects on the ruminant digestive system are not fully understood. Biochar is a carbon-rich by-product that is intended for use as soil amendment as it has been reported to increase agronomic and agricultural crop yields (Joseph et al. 2015a; Kammann et al. 2017). It has been reported that biochar applied to soils can assimilate nutrients (phosphates, organic acids and mineral complexes) and due to its recalcitrant nature, can retain and release these substances slowly over long periods of time (Kammann et al. 2017). Moreover, biochar's structure is reported to increase the water and nutrient exchange capacity in soils which could potentially promote plant growth and increase yield (Schmidt et al. 2015). In soils, it is also reported that biochar may alter and shift microbial communities which could aid in increased carbon (C) sequestration, as the physical and chemical properties of the soil are improved (Lentz et al. 2012). In terms of CH_4 in soils, a study by Feng et al. (2012) demonstrated that biochar was able to increase the methanotrophic proteobacteria, which significantly increased the ratio of

methanotrophs to methanogens. The biological mechanisms observed in soils upon biochar application are hypothesized to also manifest in animal production, as biochar may affect the bovine rumen and the associated microbes to lower CH_4 emissions and potentially improve animal productivity (O'toole et al. 2016; Kammann et al. 2017). Research to date has used a range of *in vitro* and *in vivo* experimental models to assess biochar's potential to mitigate enteric CH_4 emissions and its affects on associated parameters. However, literature that evaluates biochar in diets utilized in beef production in North America is scarce (Schmidt et al. 2019). This thesis describes four experiments that determined the effect of biochars differing in source, level of inclusion, particle size and post-pyrolysis treatment on nutrient disappearance, total gas and CH_4 production and rumen fermentation in a barley-based TMR diet typical of that offered to backgrounding beef cattle in western Canada.

LITERATURE REVIEW

Beef production in Canada

The Canadian beef industry, comprised of 62,000 beef farms and feedlots, is one of the most important sectors of the agricultural industry, contributing $18 billion annually to the GDP (Statistics Canada 2020), as well as providing high quality food and job opportunities. Alberta has the largest proportion of the Canandian cattle inventory (40.2%), followed by Saskatchewan (19.6%) and Ontario (14.1%; Statistics Canada 2020). The beef ndustry is comprised of three distinct phases; cow-calf, backgrounding and feedlot/finishing (Sheppard et al. 2015; Alemu et al. 2017). Cow-calf operations maintain breeding animals that include mature cows, breeding bulls, replacement heifers and newborn calves. Weaned calves from these operations are either retained as replacement animals or proceed to backgrounding and/or finishing operations. Cow/calf operations largely utilize pasture and forages as the basis of their nutritional regimes while cattle entering the backgrounding phase are typically offered TMR-based diets in confinement to meet the nutrient demands for growth and lean muscle deposition and to achieve desired rates of daily gain. In western Canada, barley-silage based TMR are most common for the backgrounding phase, following which cattle enter the finishing phase and are offered high grain diets (85-90% grain) to achieve the desired subcutaneous and intramuscular fat deposition (Sheppard et al. 2015; Legesse et al. 2018). The incorporation of feed additives for improved performance to backgrounding and finishing animal TMR diets is more logistically feasible than supplementation of forage in confinement or on pasture, thus the efficiacy of these supplements has been most researched in the former type of diet in Western Canada.

Greenhouse gases and global warming

In recent decades, there has been increased interest in GHGs and their impact on global warming and the environment. Over the last two centuries, the atmospheric concentrations of GHGs have dramatically increased above natural atmospheric concentrations due to anthropogenic activities (Bouchard et al. 2011; IPCC 2013). Although GHGs play an important role in maintaining global temperature, their increasing levels in the atmosphere have resulted in alterations of the ozone layer, increasing the earth's surface temperature (Nema et al. 2012). The major GHGs associated with human and agriculture activities are carbon dioxide (CO_2), nitrous oxide (N_2O) and CH_4 and they heat up the earth's atmosphere by trapping and decreasing the rate

at which energy escapes to space, acting as a blanket insulator (Podkowka et al. 2015). There are several processes in agriculture that generate GHGs including CO_2 from energy and fossil fuels, N_2O from the use of organic and inorganic fertilizers, decomposition of organic matter, volatilization and re-deposition of ammonia and nitrogen (N) leaching and CH_4 from enteric fermentation and livestock manures (Montzka et al. 2011; Hristov et al. 2013; EPA 2018). Each of the gases listed above differ in their Global Warming Potential (GWP) which describes their warming potential relative to CO_2 over a span of time, with the GWPs of 1, 28 and 296 for CO_2, CH_4 and N_2O, respectively (Environment Canada 2020). Methane, an odourless, colorless gas, has an atmospheric lifespan of 12 years compared to N_2O and CO_2, remain in the atmosphere for 170 and 230 years, respectively (Balcombe et al. 2018). Overall, anthropogenic activities contribute approximately 66.6 % of the world's total CH_4 emissions, therefore making CH_4 the second-leading climate forcer after CO_2 (Yue 2018).

Methane production in agriculture

Methane is produced naturally produced from wetlands, wildfires or from anthropogenic activities in agriculture (Malone 2015), with the latter accounting for approximately 5.6 % of global anthropogenic GHG emissions (FAO, 2017). In Canada, agriculture accounts for 8.1% of the total GHG emissions, with 40% associated with livestock production (Environment Canada 2019. Enteric livestock emissions account for 30% of national CH_4 emissions with cattle being the dominant livestock source (96%; Legesse et al. 2018; Environment Canada 2019). Other agricultural sources include manure management systems and flooding of fields which generate CH_4 from anaerobic decomposition, particularly from the treatment and storage of livestock manure in slurry systems (Malone 2015; Environment and Climate Change Canada 2017) or from excess moisture (Huang et al. 1998), respectively. Lastly, field burning is also a major source of CH_4 (Lassey 2008; Malone 2015).

Enteric CH_4 emissions in ruminants

Process of CH_4 production

Ruminants play an important role in producing human-edible products from the consumption of forages and utilization of non-arable land. This is made possible by a plethora of bacteria, protozoa and fungi that reside in the rumen that aggregate into biofilms, consisting

organized consortia enveloped in self-secreted extracellular polymeric substances and colonize feed during digestion (McAllister et al. 1994; Leng et al. 2014). These microorganisms digest forage, grains and other feeds via microbial fermentation and during this process, CH_4 is generated as a by-product. These primary digestive microbes break down ingested feeds which are made up of plant cell wall (hemicellulose and cellulose), starches, proteins and fat, into smaller, more digestible compounds such as amino acids (AA) and simple sugars (McAllister et al. 1994). These compounds are further digested by microbes through fermentation, particularly hydrolysis of simple and complex carbohydrates to yield VFAs, hydrogen (H) and CO_2. The primary site of microbial breakdown of feedstuffs is the reticulorumen, and to a lesser extent the hindgut, to produce the VFAs, acetate, propionate and butyrate, with relatively smaller amounts of valerate, caproate, isobutyrate, isovalerate and trace amounts of 2-methylbutyrate and other acids (Buddle et al. 2011). The three main fermentative pathways for the production of VFA are described below (McDonald et al. 2002; Dijkstra et al. 2005; Buddle et al. 2011):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 \text{ (acetate)} + 2CO_2 + 8H$$
$$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2 \text{ (propionate)} + 2H_2O$$
$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 \text{ (butyrate)} + 2CO_2 + 4H$$

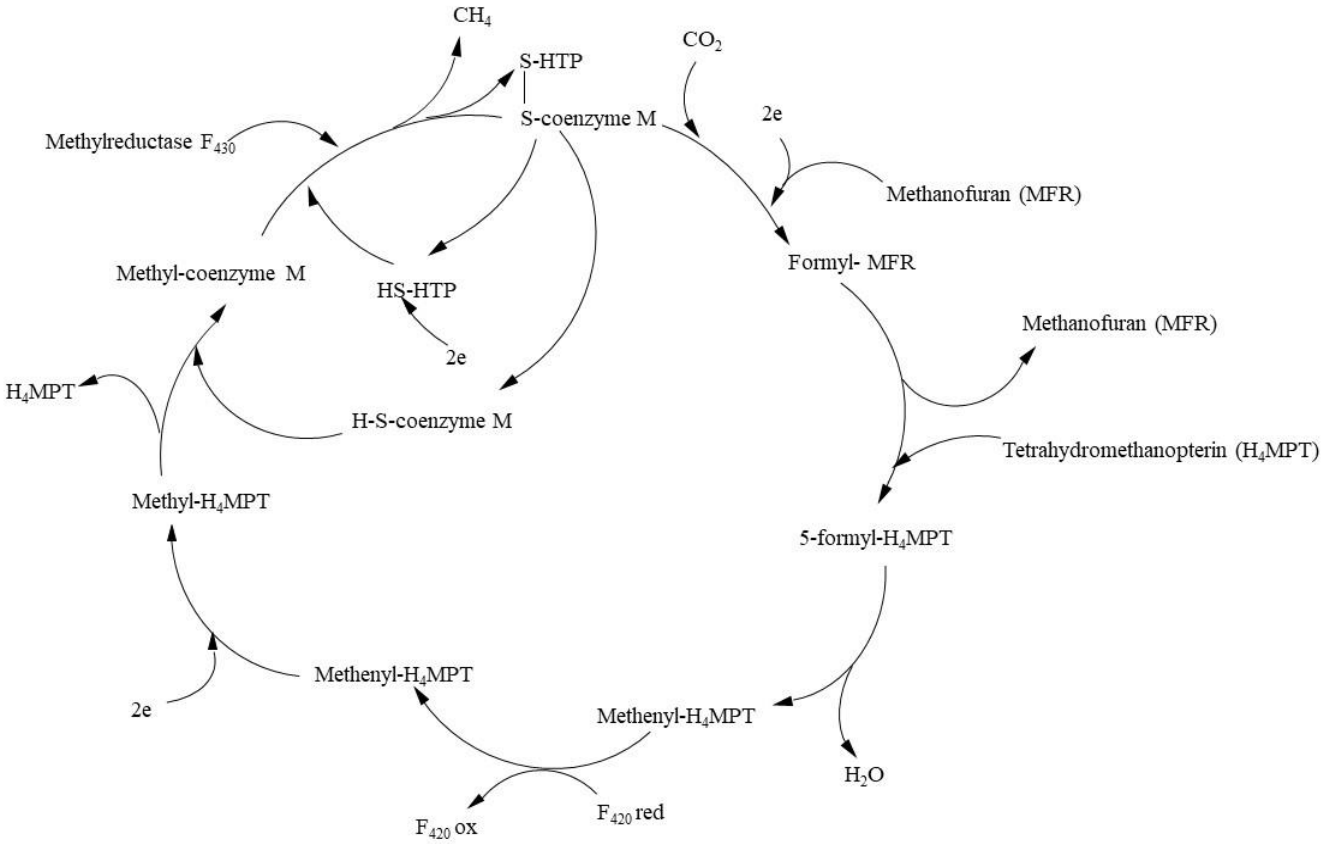
Additionally, acetate can be converted to butyrate by reduction:

$$2 \text{ acetate} + 4H \rightarrow \text{butyrate} + 2H_2O$$

The chemical composition of the ingested feed directly affects VFA production and overall gas and CH_4 production, with diets high in fiber and low in starch favouring increase in molar proportions of acetate and butyrate and a decrease in propionate which increases CH_4 production (Jayanegara et al. 2014). Starch-fermenting bacteria proliferate in high grain diets and increase the proportion of propionate, thus decreasing the overall acetate to propionate ratio, leading to decreased CH_4 production (Mitsumori and Sun 2008). The metabolic H produced in the catabolism of sugars in VFA synthesis is converted into H_2 by enzymatic activities of hydrogenase-expressing bacterial species which is consequently used for CH_4 production (Hungate et al. 1970; Whitman et al. 1992). Presence of H_2 is a vital substrate for the production of CH_4 by archaea, and the interspecies transfer of this intermediate impacts the metabolism, growth and biology of both methanogenic and methanotrophic bacteria in the rumen (Leng 2014). Excess H_2 in the rumen

inhibits fermentation reactions through negative feedback mechanisms and is theorized to reduce carbohydrate catabolism, microbial growth and microbial protein synthesis (McAllister et al. 1994; Knapp et al. 2014; Rooke et al. 2014). As described in the chemical pathways above, excess H_2 occurs during the production of acetate and butyrate and is used to reduce CO_2 to CH_4. Methane production is considered a waste product as it uses energy (2-12% of GEI; Johnson and Johnson 1995) and yields no nutritional benefit to the host, hence the production of propionate is encouraged as it acts as a competitive pathway for H use in the rumen (Bouchard et al. 2011; Buddle et al. 2011). Methanogens, the organisms responsible for the production of CH_4, become directly involved after primary digestion, utilizing the end products of anaerobic fermentation as their energy source. These organisms can produce CH_4 in several biological pathways, where they can either a) reduce CO_2 or b) cleave the methyl group of acetate by decarboxylation c) utilize formate and methylamines (Wilkinson, 2012; Patra et al. 2017). It is well established that the majority of CH_4 produced by methanogens comes from the first pathway, where CO_2 is reduced to CH_4 (McAllister et al. 1994; Jeyanathan et al. 2014) as in Figure 1.

Figure 1. Methane cycle via reduction of CO_2, adapted from the diagram of McAllister et al. (1994).



Methanogens

Methanogens are diverse hydrophobic organisms from the domain Archaea, classified into four classes, seven orders, 14 families and 33 genera (Bapteste et al. 2005; Schaechter 2009; Hackstein 2010; Patra et al. 2017). These microbes exist in varying forms and have adapted to thrive in a range of habitats including freshwater and saltwater sediments, deep subsurface rocks, hydrothermal vents in oceans, ruminants and even the human gut (Schaechter 2009). As these organisms are diverse, methanogens found in the environment differ from those that exist in animals; furthermore, methanogen species in ruminants differ from other mammalian species (Knapp et al. 2014). In ruminants, methanogens have developed symbiotic relationships with fermentative microbes and reduce the concentration of end products such as H_2, CO_2, acetate,

formate and methanol under strict anaerobic conditions to gain metabolic energy (Bouchard et al. 2011; Wilkinson 2012). These anaerobic conditions are brought about by the presence of excess H_2 resulting from the digestive activities of particle-associated microbial populations. Since large concentrations of H_2 can be potentially toxic to the rumen consortia, mechanisms serving as H_2 sinks have developed in the ruminants, of which methanogenesis is one of the most important (Lan et al. 2019). Also, methanogens are associated with ruminal ciliate protozoa indicating possible interspecies H transfer, thereby providing the necessary electrons for reducing carbon into CH_4 (Mitsumori and Sun 2008). Methanogens are also associated with aerobic rumen methanotrophic bacteria, which are a specialized group of microbes that utilize CH_4 as carbon and energy source (Parmar et al. 2015). Ultimately, methanogens derive their metabolic energy from reducing CO_2 using excess H to produce CH_4 (Johnson and Johnson 1995; Wilkinson 2012). The most dominant methanogen groups across ruminants are *Methanobrevibacter spp*., *Methanomicrobium spp*. and Thermoplasmatales-affiliated lineage C (also known as Rumen Cluster C), which recently classified as the seventh novel order of methanogenic archaea Methanoplasmatales (Paul et al. 2012). Other groups include *Methanosphaera*, *Methanomicrococcus*, *Methanosarcina* and *Methanobacterium* (Janssen and Kirs 2008). Significant hydrogenotrophic genera include *Methanobrevibacter*, *Methanosphaera*, *Methanomicrococcus*, and *Methanobacterium*, whereas less abundant methylotrophs consist of *Methanosphaera*, *Methanomasillicoccaceae* and *Methanosarcinales* (which is simultaneously methylotrophic and acetoclastic; Morgavi et al. 2010). The Global Rumen Census investigated the geographical distribution and diversity of methanogens and reported 30 species observed in cattle, sheep, bison, buffalo and a number of wild ungulates (McAllister et al. 2015). These methanogen species were diverse, with some being localized in a specific region and distribution (33% Europe, 20 % North America, 21 % Asia-Pacific, 15 % South America, 8 % Africa, and 3 % of Middle East). Common species found in ruminant digestive tracts are *Methanobrevibacter ruminantium*, *Methanosarcina bakeri*, *Methanosarcina mazei, Methanomicrobium mobile* and *Methanobacterium formicum*, with *Methanobrevibacter ruminantium* and *Methanosarcina bakeri* as the two most dominant species documented (Janssen and Kirs 2008; Patra et al. 2017; Auffret et al. 2018). These common species are similar across ruminant species (red deer, sheep and cattle) when offered diets such as pastures, silage, concentrate diets and browse (Ouwewerk et al. 2008). However, methanogen diversity is more variable in cattle and sheep offered grain-based diets compared to forage diets, likely due to

differences in ruminal pH that occur with these feeds (Ouwerkerk et al. 2008; Jeyanathan et al. 2011).

Factors affecting CH_4 emissions

In ruminants, it is well established that CH_4 production is influenced by a wide variety of factors including level of feed intake, feed type and chemical composition, animal genetics, feed conversion efficiency and other means of rumen-centric CH_4 mitigating strategies (Hristov et al. 2013; Knapp et al. 2014; Islam and Lee, 2019). A number of these strategies related to nutrition are discussed in the following sections.

Level of feed intake

Level of feed intake affects CH_4 production mainly by its impact on the rate of feed passage through the digestive tract. Generally, higher levels of feed intake result in increased CH_4 produced (% GEI) as there is more feed being fermented (Knapp et al. 2014). However, if maximum feed intake above maintenance and energy requirements of the animals are met, enteric CH_4 (g kg GEI^{-1}) is reduced due to the potential decrease in dry matter disappearance (DMD) observed at higher intakes and rates of passage (Beauchemin and McGinn 2006; Knapp et al. 2014). Passage rate affects the residence time of feed in the rumen, with slower passage rates allowing more exposure time to digestive microbes, thus increasing fermentation (Hristov et al. 2013), leadindg to more H_2 available for utilization by methanogens for methanogenesis (Knapp et al. 2014). Factors such as feed quality affect feed intake, as higher quality, more digestible feed promotes higher feed intake which consequently increases the rate of passage through the rumen (Okine, 1989; Islam and Lee 2019). Other diet characteristics that may influence feed intake and enteric CH_4 production are organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), non-fiber carbohydrates and level of concentrate in the diet (Hristov et al. 2013).

Diet chemical composition

It is well established that the chemical composition of feed plays a critical role in the production of enteric CH_4 in ruminants through its effects on feed digestibility, feed intake, ruminal pH, fermentation pathways and microbial populations (Knapp et al. 2014; Islam and Lee 2019). The pattern and rate of rumen fermentation are are largely influenced by the composition

of ingested feed dictating the pathways of VFA formation (Zhang et al. 2020). The cell wall/fiber content (cellulose, hemicellulose) of the diet plays an important role in CH_4 production as described previously, with the fermentation of fiber-based feeds promoting higher H_2 (due to the production of acetate and butyrate) reducing equivalents convert CO_2 to CH_4. Fiber in the rumen also influences protozoa populations and promotes their proliferation which may increase CH_4 production due to their symbiotic association with methanogens (Guan et al. 2006; Islam et al. 2019). The fiber content increases as the plant matures which lowers the palatability, reduces feed intake and digestibility which in turn affects the rate of passage (Hammond et al. 2013; Hristov et al. 2013). Low quality feed is suggested to increase fractional losses of energy (% GEI) lost as CH_4 , in cattle and decreasing productivity (Hristov et al. 2013; Knapp et al. 2014, Degola et al. 2016). Digestibility is often correlated with forage quality and impacts the total CH_4 emissions in dairy and beef cattle (Boadi and Wittenberg 2002). These authors evaluated impact of forage quality on CH_4, with *in vitro* OM digestibility used to characterize forage quality (i.e., high, medium, ow) and its impact on CH_4 emissions. Their results demonstrated that both dairy and beef heifers grazing higher quality pastures (crude protein (CP) 17.9; NDF, 41.8; *in vitro* OMD (IVOMD), 61.5 %) had lower CH_4 emissions (L kg^{-1} digestible OM intake) than when grazing lower quality forages (CP 11.1; NDF, 68.8; IVOMD, 50.7 %). Energy-dense diets usually contain higher levels of starch relative to NDF, which results in less CH_4 per unit of digested starch (Knapp et al. 2014). Moreover, animals offered feeds containing highly digestible vs less digestible carbohydrates at decreased intake levels results in higher fractional losses of CH_4 (% GEI) and less energy is allotted to production and growth. Conversely, higher feed intake of the same forage quality showed decreased CH_4 losses (% GEI; Johnson and Johnson, 1995). Degola et al. (2016) further supported this concept, suggesting that even though increasing feed intake increases total CH_4 production, the amount of CH_4 emitted per unit of feed intake or animal liveweight decreases. Higher quality (more energy-dense or digestible) feed provides more energy for growth and improves feed conversion efficiency as a proportion of GEI, decreasing the cost of maintenance in the animal, resulting in a decrease in CH_4 when expressed relative to liveweight or gain (Knapp et al. 2014).

Conversely, cereal grains such as those used in beef cattle feeding are rich in starch, reduce CH_4 production as H is utilized in propionate formation and diverted away from methanogenesis (Hammond et al. 2014). These highly digestible carbohydrates result in low rumen pH as they are

more rapidly digested than fibrous feeds, resulting in VFA production rates that exceed the ability of the animal to buffer them (Hammond et al. 2014; Escobar-Bahamondes et al. 2017). Low ruminal pH also inhibits the growth of methanogens and protozoa which reduces methanogenesis (Dehority 2005; Knapp et al. 2014). If protozoal populations are reduced as a result of low ruminal pH this also lowers digestion of fiber which is a CH_4 generating process. However, high starch and concentrate diets may induce sub-acute or acute ruminal acidosis hence the need to incorporate a source of long fiber in all cattle diets (Islam and Lee 2019).

The concentration of protein in the diet also affects enteric CH_4 emissions as increasing dietary CP increases the supply of N available for rumen microbial populations for protein synthesis (Van Soest 1982; Islam and Lee 2019). Degradable dietary protein is broken down into AA in the rumen and provides N for rumen microorganisms, whereas rumen non-degradable protein is utilized in the small intestine and AA are absorbed into the blood stream or total tract indigestible protein is excreted in feces (Waterman et al. 2014). Ammonia is the main N source of the rumen microbes and the availability of AA and peptides influences the proliferation of celluloytic and amylolytic bacteria (Rodriguez et al 2007). Depending on the amount of energy and protein, AA from amino acids may be incorporated into microbial protein synthesis, or deaminated into VFA production (Bach et al. 2005). Alterations to protein concentration can affect rumen N metabolism and biohydrogenation, which thereafter affects CH_4 production (Knapp et al. 2014). Ruminal protein degradation results in either net consumption or production of H_2, which in turn influences methanogenesis and/or biohydrogenation of fatty acids (FA; Knapp et al. 2014). The concentration of protein in the diet is dependent on the type and quality of feed and if dietary requirements of peptide (N/kg) and energy from OM fermented in the rumen are maximized will result in ruminal bacterial producing adequate microbial protein for utilization (McDonald et al. 2002). This is significant as rumen microbes seem to supply protein absorbed in the small intestines for maintenance, growth, pregnancy and lactation in cattle (Bach et al. 2005). This then consequently increases animal productivity which can reduce enteric CH_4 per kilogram of beef gain (DeRamus et al. 2003).

Dietary lipid concentration also affects enteric CH_4 production as lipids through biohydrogenation of unsaturated FA and inhibit protozoal activity through reduction of microbial attachment sites on feed material (McAllister et al. 1994; Knapp et al. 2014; Islam and Lee 2019). Moreover, decreased protozoa populations also results in lower fiber degradation and formation

of acetate and butyrate, hence a subsequent decrease in H_2 output for methanogenesis (Dohme et al. 2000). Lipids also decrease OM and fiber degradability and reduce substrate fermentation leading to reduced CH_4 production (Knapp et al. 2014). Polyunsaturated FA mitigate CH_4 emissions, as biohydrogenation of such acids can also serve as a H_2 sink, although to a minimal extent (1-2 % H_2 utilized via this pathway; Jenkins et al. 2008; Doreau et al. 2011). However, it is recommended that lipid concentration not exceed 6-7% of dietary DM as this may negatively impact feed intake, particularly fiber digestion (Beauchemin et al. 2008). Additionally, the negative effect of lipid supplementation on enteric CH_4 has been recognized as an effective strategy in high grain and TMR-based diets (Grainger and Beauchemin 2011); however, little literature exists for the use of lipids in forage-based diets due to the difficulty in supplementation in grazing and forage-fed scenarios

Forage type

The type of forage influences the extent of CH_4 emissions in cattle, with the CH_4 potential of perennial and annual forages and cereal grains/by-products differing significantly. Perennial forages such as grasses and legumes are often incorporated in cattle production particularly in the cow-calf phase where grazed pasture makes up a significant portion of the diet. Compared to cattle offered perennial grasses, the CH_4 emitted from legume fed animals may be lower (McCaughey et al. 1999) but this is not always the case as this could also be affected by other factors such as DM intake and fiber content (Van Dorland et al. 2007). The associated reduction in enteric CH_4 are related to high condensed tannins (CT) which may be present in some legumes (sainfoin, birdsfoot trefoil) but not in all (alfalfa). The inclusion of these forages in diets to mitigate CH_4 production are not only due to their CT but also their lower fiber content (Beauchemin et al. 2008). The lower fiber content increases digestibility, rate of passage and decreases ruminal fermentation, thereby decreasing CH_4 production. Further, the CT content in these forages may decrease rumen protein degradation (and lower H_2 production) as well as inhibit protozoa and methanogen activity which may decrease methanogenesis (Patra 2010). However, Chung et al. (2013) reported that heifers offered fresh sainfoin hay-based diets which differed in the proportion of sainfoin (20 vs 100 %; extractable CT 0.55 %) did not differ in enteric CH_4 emissions, suggesting that there may be a threshold of CT content below which CH_4 emissions are not affected. Additionally, cereal grains and silages are highly digestible providing starch when incorporated into cattle diets which

reduces enteric CH_4 by several mechanisms as described previously. Due to relatively high digestibility cereal/grain silages, feed intake may be increased relative to perennial forages, thus reducing the rumen fermentation time due to increased passage rates in the rumen (Beauchemin et al. 2008). However, the intrinsic differences between grains and cereal silages such as starch content and degradable fiber factions may influence the relative abundance of rumen protozoa and the overall biofilm microbial population, favoring propionate or acetate producing bacteria which affects end products of VFA fermentation; therefore, this consequently affects fermentation VFA and gases (Beauchemin and McGinn 2005; Lengowski et al. 2016). Beauchemin and McGinn (2005) compared CH_4 (g kg DMI^{-1}) production in growing beef cattle offered corn or barley-based diets and reported lower CH_4 emissions with corn compared to barley in the finishing stages. The corn and barley diets did not differ in digestibilities but resulted in higher total VFA (decreased proportions of acetate and increased propionate) and lower ruminal pH, which likely inhibited methanogenic bacteria causing decreased CH_4 emission. Additionally, Benchaar et al. (2014) reported a decline in CH_4 production (% GEI) when an increasing proportion of corn silage in TMR diets replaced barley silage as offered to dairy cows. Moreover, these authors observed an increase in feed intake as corn silage increased in the diet and reported higher ruminal OM degradability with increasing proportion of propionate and decreasing acetate, which may have caused the overall reduction in enteric CH_4. Molar proportions of propionate increased as compared to acetate, which thereby reduced CH_4 production.

Supplementation of feed additives

Enteric CH_4 emissions are influenced by a variety of dietary factors, thus nutritional manipulation is of central importance to mitigation. The effects of supplementing novel feed additives such as plant secondary compounds, organic acids and chemical inhibitors on enteric CH_4 emissions from ruminants has been an area of extensive research in recent years. However, their potential is dependent on the intake, quality and type of basal diet and for animals on pasture it is logistically challenging to include supplements in the diet. Thus, the use of these mitigation strategies is generally limited to backgrounding and finishing phases. Feed additives are often supplemented in small amounts rather than replacing major diet ingredients and can affect enteric CH_4 emissions in a number of ways such as improved rumen fermentation, alterations in VFA profiles, defaunation, direct inhibition of methanogens, or substitute electron receptors/H_2 sinks

(Mitsumori et al. 2012; Hristov et al. 2013). Saponins present in forages and also potential supplements to the diet affect fermentation by defaunation, inhibiting protozoal activity by binding sterols in their membranes, causing lysis (Bouchard et al. 2011). As protozoa are competitors and predators of ruminal bacteria, the detrimental effect of saponins on protozoa favours bacterial growth (Patra 2010). Consequently, the methanotrophic to methanogenic bacteria ratio would increase, thus reducing CH_4 emissions. The effects of saponins, however, are variable and can be partially attributed to source and level as reported in a meta-analysis by Jayanegara et al. (2014) who indicated saponin-rich sources either decreased (Holtshausen et al. 2009) or had no effect (Staerfl et al. 2010) to CH_4 emissions in diets *in vitro*. The transient effects of saponins may be attributed to the possible proliferation of saponin-degrading microbes, and this in the long run can limit its practical use (Hart et al. 2008).

Organic acids, including lauric acid, myristic acid, linseed oil and fumarate (electron receptor) increase the molar proportion of propionate, resulting in a decrease in CH_4 production in lactating dairy cows (van Zijderveld et al. 2011); however, this is not consistent as organic acids have elicited no effects on CH_4 production of beef heifers (Beauchemin and McGinn 2006) and dairy cattle (McCourt et al. 2008). The addition of ionophores, which is a commonly used feed additive to improve feed efficiency in beef cattle, has also CH_4 mitigation potential (Hristov et al. 2013). Ionophores are toxic to gram-positive bacteria, protozoa and fungi, which can be attributed to their ability to penetrate through biological membranes and interfere with the transport of ions across cellular membranes (Beauchemin et al. 2008; Ellis et al. 2012). It is reported that ionophores can decrease H_2 producing protozoa which, thereafter, inhibits H_2 transfer for methanogenesis (Beauchemin et al. 2008; Vyas et al. 2018). Protozoa also are sensitive to the dosage of ionophores (Guan et al. 2006; Ellis et al. 2012) an outcome that may result in a with decrease in enteric CH_4 via defaunation at high (33 mg kg^{-1} diet DM) levels of inclusion. However, the effect of ionophores on CH_4 suppression are reported to be short-lived as studies show that CH_4 returns to baseline levels after a period of time (Saa et al. 1993; Guan et al. 2006; Knapp et al. 2014).

Electron acceptors such as nitrates, sulfates and fumarates have also been supplemented in beef cattle diets to inhibit CH_4. Nitrate-based compounds are reported to block methanogenesis and formate synthesis (Leng 2014) and a reduction in CH_4 has been reported *in vitro* and *in vivo* (Anderson et al. 2010; Madsen et al. 2010; Hulshof et al. 2012). Additionally, the inclusion of nitroethane can increase molar proportions of propionate and butyrate, reducing CH_4 emissions

(Anderson et al. 2010; Lee et al. 2017). Nitrates and sulfates can also lower CH_4 production, as the reduction of these compounds in the rumen can serve as an alternative H_2 sink and divert H_2 from methanogenesis (Leng 2014; Lee and Beauchemin 2014). Cattle supplemented with increasing amounts of nitrate (replacing urea) exhibited a progressive decline in enteric CH_4 emissions (Hulshof et al. 2012), with the inclusion of sulfate reducing CH_4 production in sheep (van Zijderveld et al. 2010). However, nitrate and nitrite (from nitrate reduction) long term may affect microbial groups and can pose inhibitory effects of cellulolytic and xylanolytic bacteria (Marais et al. 1988; Iwamoto et al. 2002). Fumarate reduced CH_4 production when coupled with organic acids in lactating dairy cows (van Zijderveld et al. 2011) as it may also serve as a H_2 sink in the rumen (Bayaru et al. 2001; Garcia-Martinez et al. 2007). The highest CH_4 reduction were elicited from the addition of fumarate to high (CP, 13.5 %; NDF, 38.7 %; OM, 94.7 %) vs low forage diets (CP, 15.6 %; NDF, 20.8 %; OM, 96.6 %) *in vitro* (Garcia-Martinez et al. 2007), but is transient *in vivo* (Lopez et al. 1999) as fumarate positive effects seem to be influenced by its level of inclusion and the type of fermented substrate (Garcia-Martinez et al. 2007). However, the supplementation of these electron receptors are regulated or often supplied with direct-fed microbials so as to reduce toxic intermediate metabolites more rapidly and mitigate potential adverse effects (Lee and Beauchemin 2014; Lee et al. 2017). Overall, despite the promising results of lowered CH_4 emissions, feed additives often have transient and variable effects, thus their long term application is unclear.

Activated carbon and carbon-based such as biochar have been recently included in animal diets as various researches suggest the potential of these products in improving animal health without negatively impacting rumen chemistry and functionality (Van et al. 2006; Gerlach et al. 2014; Pereira et al. 2014). Moreover, carbon by-products have been shown to decrease CH_4 emissions and many speculations on how so are theoretically explained by a number of mechanisms. Notwithstanding, the application of any proposed or novel CH_4 mitigation strategy should be accompanied by an assessment of the whole system GHG output to ensure that reductions in CH_4 are not accompanied by increases in other gases or adverse effects to animal welfare and performance.

Biochar: origin, production, composition and application

Biochar origin and production

Biochar is a term coined from the words "bio" and "char" from charcoal and it is a carbonaceous by-product derived from pyrolysis of biomass (i.e., forages, wood, straws, manure and agricultural wastes) in low or zero oxygen (O) environments ranging of 400-1000 °C. Pyrolysis is a series of irreversible thermochemical steps that decompose material and lead to the conversion of biomass C into a more recalcitrant, persistent structure (Lehmann and Joseph 2012; McFarlane et al. 2017). Pyrolysis is not a new process as biochar production technologies are partly based on the processes which occur during forest fires that convert woody and organic biomass into partly combusted, pyrolyzed carbonaceous material that contributes to mineral enrichment within soils. Such residues are naturally formed and found, for example, in the Amazonian forests of South America, derived from burning biomass as practiced by old Amerindian populations centuries ago (Lehmann and Joseph. 2012). Charcoal is also produced via pyrolysis, but some key differences between charcoal and biochar are a) end product use b) source c) pyrolysis temperature (Man et al. 2020). Charcoal is used as a fuel for cooking and energy, whereas biochar is often used in the environment for pollutant adsorption or for soil amendment. Charcoal is usually prepared from high-energy containing woody biomass, whereas biochar from a range of materials, such as agricultural residues, organic wastes, and woody biomass. The pyrolysis temperature of biochar (350 – 1000°C) is usually higher than charcoal (105 - 950°C) as to develop the porosity to enhance adsorptive capacity. With biochar's unique biochemical ability to assimilate and retain nutrients and minerals, as it has the ability to adsorb cations per unit of carbon due to its surface area, greater negative surface charge and charge density (Lehmann and Joseph 2012). This property made biochar specifically mass produced as a soil amendment for agronomic and environmental management purposes and also acts as an important pre-cursor to activated carbon as it aids in the release of minerals and adsorbs toxicants from the soil (Barrow 2012; Quin et al. 2015). Biochar is converted into activated carbon by a series of physical (CO_2, steam) or chemical (salt, organic and inorganic acid treatment) activating steps which are applied to increase its adsorptive capacity (Devi and Saroha, 2016).

Biomass sources ranging from manures, agricultural residues and lignocellulose rich sources such as wood and straws are used to produce biochar. Pyrolysis of biomass results in biochar products varying in biochemical properties and nutrient profile depending on the source

material and production conditions. The half-life of recalcitrant C in biochar is approximately 1000 years with the decomposition and degradation of such carbon being significantly slower than uncharred carbon from organic sources due to the presence of more aromatic carbon which persist in the environment longer than other forms of organic carbon (Lehmann and Joseph 2012). Biochar is usually regarded as a by-product intended to be a pre-cursor of activated carbon used in purification processes (Horne and Williams 1996). In biochar production, pyrolysis includes a variety of exothermic processes and release gases and heat along with other valuable compounds (liquid bio oil, syn-gas) and by-products (Czernik and Bridgwater 2004), with the conditions during the process dictating the biochemical properties of the biochar produced (Lehmann and Joseph 2009). Currently, biochar in Canada is listed as a primary supplement material deemed by the Canadian Food Inspection Agency (CFIA) safe, in compliance to regulatory and labelling requirements under *Fertilizers act* and *Fertilizers regulation* (Government Canada, 2020). However, biochar can also be incorporated in feed in amounts not exceeding 250 g tonne^{-1} of the complete feed (LRDC-CFIA, personal communication).

Biochar physiochemical characteristics

Biochar mainly consists of four components, which are stable C, labile C, ash and moisture. Among these components, the stable C is the most abundant, whilst the least abundant is ash/mineral (Verheijen et al. 2010). The proportions of these depends on a variety of factors such as the origin of biomass, pyrolysis parameters (temperature, pressure, heating rate) and post handling/treatment post pyrolysis (Downie et al. 2009). The origin of biomass determines the presence of aliphatic (saturated or unsaturated, straight chained, branched or cyclic hydrocarbons) and more volatile compounds in the biochar. Biochar rich in volatile matter (phenols, glucopyranoses, cyclopentones) can be derived from woody feedstock rich in lignin, hemicellulose and cellulose when produced at relatively lower temperatures (400°C; Jindo et al. 2014; Bhattacharya et al. 2015). At higher temperatures (> 600°C) woody biochars tend to contain less labile elements compared to biochars derived from agricultural residues as the former have relatively higher amounts of aromatic compounds (Jindo et al. 2014). The more labile that the aliphatic components are, the more rapidly they mineralize compared to stable C in biochar compounds (Lehmann and Joseph 2009). The physical stability of biochar is not only due to the abundance of stable C but also due to the mineralization of labile C that provides a physical

protection to the biochar. This phenomenon is heavily favoured by biochar's particulate form, which when mineralized, restricts decay and oxidation in the outer areas of the particle (Lehmann and Joseph 2009). This is evident in naturally formed biochar in the Amazonian forests, which have persisted over thousands of years (Lehmann and Joseph 2009). However, quantitatively it is not yet known if the ability of biochar to form complexes with minerals contributes to its stable composition (Schmidt and Noack 2000; Lehmann and Joseph 2009). Generally, commercially produced biochar exists in different particle shapes and sizes depending on the conditions of pyrolysis and the subsequent post treatment and handling.

According to Amonette and Joseph (2009), the optimum pyrolysis temperature necessary to achieve the greatest biochar yield range from 300 to 600°C, resulting in products that are more porous, carbonaceous and stable. The higher the firing temperature (maximum temperature attained in pyrolysis), the more porous the final by-product (Lehmann and Joseph 2009). Lower temperature and slower heating rates yield higher amounts of biochar, while higher temperature pyrolysis with faster heating rates (or gasification) results in lower amounts or no biochar at all (Gaojin and Shubin, 2012). Temperature also determines the particle size in biochar production and as increasing temperature leads to decreased particle size associated with rapid decomposition of volatile material (Verheijen et al. 2010). Stability may be influenced by aromaticity, and biochar can be classified in terms or C aromaticity, whether with an abundance of C stacked into graphene sheets (conducting phase) or with less C ordering, with more complexes of aromatic and aliphatic compounds (non-conducting phase) and inorganic ash. With the formation of these graphene sheets, the solid density of biochar also increases ultimately strengthening the biochar's physical integrity and stability. These graphene sheets also result in increased surface area per volume despite the decline in particle size and these single layers of polyaromatic structures exhibit good electrical conductivity and renders the recalcitrant and stable nature of biochar (Geim and Novoselov 2007; Chacon et al. 2017). In addition to C, biochar contains other elements such as H, N, O, sulphur (S) and Phosphorus (P). The ratio of these elements (particularly O and H) relative to C are used to measure aromaticity and maturation, whereas N, P and S with other functional groups determine the biochar's surface charge (Hammes et al. 2006). Studies have shown that as pyrolysis temperatures increase, C content proportionally increases while O and H decrease (Krull et al. 2009; Domingues et al. 2017). The processing of biochar influences its physical characteristics as well as its chemical attributes. The pH of biochar can vary, ranging from slightly

acidic to alkaline, which may be attributed to the variation in pyrolysis temperatures and feedstock biomass (Van Zwieten et al. 2015). As the pyrolysis temperature increases, pH tends to increase as the association between cationic compounds increases (Van Zwieten et al. 2010). Alkaline functional groups such as carbonates, oxides and hydroxides are enriched due to the latter's increasing abundance (Domingues et al. 2017) and the presence of carbonates is the main determinant of biochar's alkaline nature.

Post pyrolysis, biochar becomes porous in nature and is characterized by high surface area, similar to activated carbon. The increased porosity impacts the chemical properties of biochar as the formation of micropores increases the surface area and improves its adsorptive properties, enabling it to absorb liquids, gases, nutrients, minerals and even toxins (Lehmann and Joseph 2012). Biochar, when applied in soils has the tendency to absorb more cations per unit C than other forms of organic C (Sombroek et al. 2003) and it also appears to have a strong affinity for the anion phosphate, recycling P and its retention in biochar surfaces being slowly released, providing a continuous P; this in addition to the binding of compounds and cations, can be essential for plant growth (Lehmann and Joseph 2009). This may be further explained by the presence of several components in the surface (micropores) and the graphene sheets within the biochar structure (Chacon et al. 2017).

Biochar biological properties

The different functional groups of various elements, organic molecules, amorphous and labile C determine overall physiochemical properties of biochar, and are key factors distating interactions when added as a soil amendment affecting soil environment and thereby affecting various root traits such as biomass, microorganisms, as well as soil OM and nutrients (Van Zwieten et al. 2015). These interactions form organo-mineral-biochar complexes, as biochar can be either a donor or acceptor of electrons (Hammes and Schmidt 2009; Leng 2014). Redox reactions are crucial in biological reactions, nutrient uptake, transformation and other biochemical processes in plants, root systems and microbial communities (Thies and Rillig 2009). These biochemical reactions, both biotic and abiotic, occur on the surface and in the pores of biochar (Lehmann and Joseph 2012). Biochar can facilitate direct interspecies electron transfer within microbes in a community due to its conductivity and electrochemical properties (Leng 2014), coupling redox activities (Chacon et al. 2017). These redox reactions occur in the soil-rhizosphere-plant systems

and critically affect cell and plant physiology, microorganism structure, nutrient uptake among other processes and biochar can interact with this system to form organo-mineral-biochar complexes (Joseph et al. 2015a; Chacon et al. 2017).

In addition to its intrinsic characteristics, the physical attributes of biochar also play a role in forming biotic relationships with living systems. The presence of micropores, as well as its high surface area, makes biochar an excellent adsorber of different organic nutrients, inorganic compounds and gases (Cayuela et al. 2014), creating a suitable habitat for microorganisms to colonize and reproduce (Lehmann and Joseph 2009). In addition, bacteria can grow in biochar-rich environments as they aid in microbial attachment and provide physical protection within the micropores (Bhattacharya et al. 2015). Overall, the interplay of biochar's physical and chemical characteristics plays a major role in different living systems by influencing the different metabolic enzymes and processes, availability of different nutrients and environmental conditions such as pH.

Biochar application in agriculture

Biochar is used as a soil amendment due to its adsorbing properties which are influenced by the available surface area, pore size distribution and surface chemistry of the material (Lehmann and Joseph 2012; Bhattacharya et al. 2015; Chacon et al. 2017). Biochar tends to improve nutrient cycling and soil structure by increasing the soil organic content as it contributes a significant amount of C (Hammes and Schmidt 2009). Moreover, biochar demonstrates high stability against soil decay and can retain nutrients, compounds and form mineral complexes (Lehmann and Joseph 2009). Thus, it has been suggested that with these properties, biochar may potentially alleviate environmental pollution and improve overall soil quality (Quin et al. 2015; Domingues et al. 2017). More specifically, Mohan et al. (2007) have reported that wood-based biochars (oak and pine) have the potential to remove lead, cadmium and arsenic in water, and in soils, Steiner et al. (2004) reported that biochar enhances soil structure and water retention ability, promotes nutrient mobility and reduces aluminum toxicity. Biochar is also reported to be able to remove and mitigate GHG in soils (Barrow et al. 2012; Feng et al 2012; Quin et al. 2015) as it can decrease N_2O and CH_4 emissions by changing soil physical properties (gas diffusivity, aggregation and water retention) as well as chemical properties (pH, availability of organic N and dissolved C) and shift microbial

populations in the soil (increase in denitrifiers and methanotrophs) that may reduce sthese GHG emissions.

Biochar as feed additive in cattle and other livestock

The use of charcoal has been increasingly prevalent from late 19th/early 20th centuries to improve animal performance and health (Savage 1917; O'toole et al. 2016) and has been used as medication to treat viral and bacterial infections in livestock (Schmidt et al. 2016). Biochar offered to cattle, sheep, pigs and poultry has shown positive effects on productivity and health parameters such as digestion, feed utilization efficiency, weight gain, milk somatic cell count, toxin and gas adsorption and blood values, but responses are inconsistent (Schmidt et al. 2016; Kammann et al. 2017). Improved digestion and weight gain may be attributed to an overall increase in pH in the animal gut, as biochars are generally alkaline in nature (Kammann et al. 2017). This may buffer acidic gut pH, preventing conditions, such as acidosis in cattle, which are known to impact weight gain and productivity. Biochar's buffering capacity was exhibited in anaerobic digestion of complex organic wastes and was reported to mitigate pH decrease by promoting oxidation of butyrate under high H_2 partial pressure by acting as a temporary electron acceptor (Wang et al. 2018).

Biochar, when supplemented with organic acids can decrease populations of *Cryptosporidium* in bovine calves (Watarai et al. 2008). This is a result of the chemical reactions that occur when biochar, in addition to wood vinegar, altering the O and N functional groups present in the gut and releasing labile organic substances that act as biocides owing to their which acidic nature (Watarai et al. 2008). Moreover, in a meta-analysis of cattle studies, Schmidt et al (2019) report that inclusion of biochar in the diet may increase growth rates in dairy and beef cattle, and is speculated to enhance the reactivity of biochar surfaces when exposed to the GIT, where acid-base reactions continuously occur, which may aid in the formation of functional groups that can enhance microbial redox reactions (Klüpfel et al. 2014; Schmidt et al. 2019). The formation and release of organic functional groups in the animal's digestive system not only promotes the adsorption of hydrophobic (fungal) and pathogenic toxins, hypothesized to be adsorbed through binding sites in pores, or binding the pathogens immobilizing their activity (Schmidt et al. 2019). These mechanisms are influenced by the functional groups in the biochar surfaces as well as pore size (Clark et al. 1998; Watarai et al. 2008; Schmidt et al. 2019). Biochar-

supplemented feed, for instance has been shown to alleviate botulism symptoms in infected Holstein cows with reduced levels of *Clostridium botulinum*, as well as neurotoxins, bacterial proteins and enzymes, detected in fecal samples (Gerlach and Schmidt 2014a; Gerlach et al. 2014).

Biochar also promotes the conversion of organic substances (carbohydrates, sugars and proteins) and elements into more mobile forms, such as reduced element states and mobilized nutrients (P, Ca, K, N, Mg), that are more available to the animal for absorption and utilization as mobilization mechanisms were observed in soils (Joseph et al. 2015b). The redox mediating capabilities of biochar is speculated to facilitate the formation of functional and syntrophic rumen consortia, and this would improve the efficiency between microbe interactions which would lead to enhanced feed conversion eficiency (Leng et al. 2012a, b, c; Leng 2014). In these living systems, activated biochar is speculated to adsorb signaling compounds that may able to change gene expression and structures of such microbial communities, though further research is needed in this area (Ermolaeva et al. 1999; Masiello et al. 2013).

However, overall effects of biochar in cattle are not always positive as demonstrated by a study of Terry et al. (2020) who reported a tendency for reduced average daily and total weight again in steers fed biochar at up to 2% of DM, with no effect on feed intake, gain-to-feed ratio and net energy gain. This may be due to the inert nature of biochar and being unmetabolizable in the rumen, therefore not affecting ruminal metabolism and fermentation (Terry et al. 2019b). This was further demonstrated in studies by Terry et al. (2019b) and Winders et al. (2019) who reported that biochar did not affect intake of beef cattle offered high-forage or high-grain diets.

Biochar has been shown to reduced pathogenic species such as *Campylobacter sp.* in layer chickens, which is a promising breakthrough in controlling zoonotic diseases that may be transmitted amongpoultry (Prasai et al. 2016). Gerlach and Schmidt (2014b) also reported that biochar improved hygiene as the incidence of pathogenic bacteria and mycotoxins were reduced when added as litter amendment in chicken coops. With it's adsorbing capacities, biochar can lock in excrement moisture, inorganic and organic N which could lower ammonia emissions (Gerlach 2014b).

Biochar and GHG mitigation

With its adsorptive and and electron shuttling capabilities, biochar has been reported to retain and remove several GHG from soils, such as N_2O, CO_2 and CH_4 (Feng et al. 2012; Cayuela

et al. 2014; Kammann et al. 2015). However, these mechanisms may not be only attributed to biochar but also to soil structure and biochemical properties (pH, organic content, soil type; Kammann et al. 2017). These properties may affect sorption interaction effects of biochar with inherent microbial populations in the environment (Cayuela et al. 2014; Schimmelpfennig et al. 2014). In rice paddy soils (populated with methanogenic archaea), biochar has shown inconsistent trends with regard to the amount of total gas and CH_4 produced as it has been reported to increase (Yu et al. 2013), decrease (Feng et al. 2012), or not impact emissons (Xie et al. 2013) upon application. Biochar's mitigating effects on CH_4 is more prominent on anoxic soils as methanotrophic organisms have been observed to consume and oxidize CH_4 more efficiently (Feng et al. 2012). The same trend has been observed in rice paddy soils, where CH_4 effluxes have been reduced due to the addition of electron-accepting ash-rich material and sewage sludge biochar (Reddy et al. 2014).

In animal production, as discussed previously, the mechanism of biochar mitigation of GHG is due to its ability to absorb compounds and its influence, directly and indirectly, on the microbial communities in the gut (Kalachniuk 1994; Lehmann and Joseph 2009; Joseph et al. 2015b; Kammann et al. 2017). A study by Leng et al. (2012c) suggests that biochar inclusion decreased CH_4 production (ppm) as a result of improved feed conversion efficiency and productivity, as well as potentially favoring the formation of methanotrophic consortia in biochar surfaces potentially leading to increased CH_4 oxidation and lower emissions. The methanotrophic to methanogenic ratio in the rumen was hypothesized to increase with biochar addition, as has been demonstrated in biochar-amended soils (Feng et al. 2012). The interaction between synthrophic and biofilm microbial populations, eventually leading to increased efficiency in ATP production and utilization, as well as the promotion of methanotrophs, would potentially favor anaerobic CH_4 oxidation (Leng et al. 2012a, b, c). Saleem et al. (2018) reported that biochar decreased CH_4 (expressed as % of total gas; mg per day; g/kg DM incubated and DM digested), in a barley silage-based diet fed to an artificial (RUSITEC) rumen system. However, contrasting results were observed in several studies *in vitro* (Hansen et al. 2012; Pereira et al. 2014; Cabeza et al. 2018; Teoh et al. 2019) and *in vivo* (Terry et al. 2019b; Winders et al. 2019) using biochar products differing in source and levels of supplementation. The differences in source material and heating temperature during pyrolysis was suggested to influence the adsorptive capacity of biochar, hence possibly gaseous emissions (Lehmann and Joseph 2009; McFarlane et al. 2017;

Cabeza et al. 2018). As a result, further investigation of biochar supplemented to cattle diets are warranted to assess its role as a feed additive to reduce enteric CH_4 emissions.

Summary and conclusions

Agricultural activities generate GHGs from a variety of sources, with CH_4 being one of the major contributors from cattle production as a by-product of ruminanl fermentation. As a result, public concerns regarding the carbon footprint of beef production remains an ever-present consideration for the industry. Enteric CH_4 production is predominantly affected by nutritional parameters (level of intake, type of feed, diet composition, supplementation and use of additives). A novel dietary approach to potentially mitigate CH_4 emissions is the incorporation of biochar, a carbon-rich by-product obtained by the pyrolysis of biomass material, into beef cattle diets. Studies have shown that it is an effective soil amendment, decreasing concentrations toxicants, GHGs and heavy metals by altering the microbial populations present in the soil. Several studies have shown that these adsorptive properties are also present when biochar is included in animal diets, suggesting biochar potential may play a role in improving animal productivity. However, this is dependent on biochar's inherent physical and chemical characteristics influenced by conditions during pyrolysis and data to date has not been conclusive. Additionally, a gap in the literature exists regarding the evaluation of biochar in TMR-based diets typical of those offered to beef cattle in western Canada. Thus, the objective of this thesis was to evaluate the potential of biochar to mitigate CH_4 and improve digestion and rumen fermentation in a barley silage-based TMR-based diet using *in vitro* methodologies.

HYPOTHESES AND OBJECTIVES

Hypotheses

The overall hypothesis of this thesis is that the addition of biochar to a barley silage-based TMR *in vitro* will decrease enteric CH_4 emission and improve feed digestion and fermentation. The specific experimental hypotheses are as follows:

Chapter 1: The addition of biochar to a barley silage-based TMR *in vitro* will decrease CH_4 production (mL/g DMD) and improve feed digestion and fermentation. Moreover, increasing levels of biochar and decreased fine particle size will lower CH_4 production and improve feed digestion and rumen fermentation.

Chapter 2: The addition of biochar to a barley silage-based TMR in a RUSITEC system will decrease CH_4 production (mg/g DMD) and improve feed digestion, fermentation and increase rumen biofilm microbial populations.

Chapter 3: The addition of biochar differing in post-pyrolysis treatment to a barley silage-based TMR in a RUSITEC system will decrease CH_4 production (mg/g DMD) and improve feed digestion, fermentation and rumen biofilm microbial populations.

Biochar is hypothesized to aid in CH_4 mitigation in ruminant diets through several mechanisms including its ability to i) alteri and shift microbial communities to decrease CH_4 emissions ppm; Leng 2014); ii) serve as an alternate electron acceptor and divert H_2 away from methanogenesis which has been demonstrated *in vitro* (Leng et al. 2012a, b, c); iii) increase the methanotrophic proteobacteria, which significantly would increase the ratio between methanotrophs to methanogens (Feng et al. 2012); iv) affect interactions between microbial populations and improve biofilm formation, increasing substrate digestibility (Leng et al. 2014; Kammann et al. 2017; Saleem et al. 2018) and decreasingCH_4 emissions. Therefore, these associated beneficial effects of biochars in soil potentially demonstrate a novel approach for dietary manipulation when added to cattle diets.

Objectives

The objectives of this thesis were:

Chapter 1: To determine the effects of seven biochar sources, supplemented at two inclusion levels and three particle sizes on *in vitro* DMD, total gas and CH_4 production and fermentation parameters in a barley silage-based TMR diet.

Chapter 2: To determine the effects of three pine-based biochar differing in physiochemical properties on nutrient disappearance, total gas and CH_4 production, fermentation parameters and rumen microbiota in a RUSITEC system fed a barley silage-based TMR.

Chapter 3: To evaluate the effects of three spruced-based biochars differing in post-pyrolysis treatment on nutrient disappearance, total gas and CH_4 production, rumen fermentation and rumen microbiota in a RUSITEC system fed a barley silage-based TMR.

CHAPTER 1

Effects of biochar source, level of inclusion and particle size on *in vitro* DMD, total gas and CH_4 production and ruminal fermentation parameters in a barley silage-based TMR diet

Abstract

This study evaluated the effects of biochar differing in source, level of inclusion, and particle size on DM disappearance (DMD), total gas and methane (CH_4) production, and ruminal fermentation in a barley silage-based diet. The seven biochar products used were coconut (CP001 and CP014) or pine (CP002, CP015, CP016, CP023, CP024)-based. Experiment 1 evaluated these biochars at 4.5, 13.5 and 22.5% level of diet inclusion, whereas Experiment 2 evaluated CP002, CP016 and CP023 at 2.25 and 4.50% of the diet at < 0.5, 0.5-2.0, > 2.0 mm particle size. Data were analyzed using PROC MIXED in SAS as a randomized complete block design, with biochar source, level of inclusion and particle size (Exp. 2 only) as fixed effects with run and replicate as random effects. Increasing level of biochar inclusion linearly ($P < 0.01$) decreased DMD in Exp. 1, with no response ($P > 0.05$) in Exp. 2. Total gas, CH_4 (mL/g DMD) and rumen fermentation parameters were not affected by treatment, level of inclusion or particle size ($P > 0.05$). In conclusion, biochar of varying source and particle size did not mitigate CH_4 emissions, but reduced DMD at higher levels of inclusion in the barley silage-based TMR diet.

Introduction

Globally, the agricultural sector contributes 26 % of anthropogenic greenhouse gases (GHG), mainly CO_2, N_2O and CH_4 which are the subject of increased scrutiny owing to their contribution to global warming and climate change (Alemu et al. 2017). Of this total, 5.6% can be attributed to enteric CH_4, with cattle being the dominant source from livestock (FAO, 2017; Frank et al. 2017). Methane is a natural by-product of digestion in ruminants as feed material is broken down by a plethora of rumen microbes via microbial fermentation (McAllister et al. 1994; Johnson and Johnson 1995). Furthermore, enteric CH_4 production is primarily influenced by the chemical composition of the feed and its impacts fermentation pathways and the fate of reducing equivalents in end products (Buddle et al. 2011; Hansen et al. 2012).Thus, rumen-centric strategies (such as dietary manipulation) have been examined with the objective of mitigating CH_4 production while improving animal productivity (Martinez-Fernandez et al. 2016; Haque et al. 2018). Hence, there has been a significant and growing interest in the inclusion of novel feed additives in ruminant diets (Terry et al. 2018).

Biochar is a carbon-rich by-product produced from the manufacturing of activated carbon, which is used in industrial and soil purification processes (Horne and Williams 1996). Biomass

sources of biochar include manures, agricultural crop residues and lignocellulose rich sources such as wood and straws. It has been applied to soils as an amendment to aid in the slow release of minerals and adsorb toxicants (Barrow 2012; Chacon 2017). It has been suggested that biochar can mitigate soil CH_4 emissions by increasing the ratio of methanotrophs to methanogens (with biochar also having potential inhibitory effects on methanogens) leading to increased CH_4 oxidation and lowered CH_4 emissions (Feng et al. 2012; Leng 2014). It has been proposed that in manure and soil, the structure and porosity of biochar mediate electron transfer among bacterial species (Chen et al. 2008; Kammann et al. 2017). In the rumen, it may act as a digestive catalyst, promoting biotic to abiotic assemblages that enhance microbial fermentation. This is achieved as biochar can be solid interphase mediating electron transfers between microbes and terminal acceptors (Watarai et al. 2008; Leng et al. 2012a, b, c; Mitsumori et al. 2012). Moreover, due to its electron mediating capabilities within microbial communities, biochar may lower enteric CH_4 by shifting ruminal fermentation towards propionate production, which acts as an alternate H_2 sink to the reduction of CO_2 to CH_4 by methanogens (Mitsumori and Sun 2008; Mitsumori et al. 2012, Leng et al. 2014).

However, the effectiveness of biochar as an additive in animal feeding systems to mitigate CH_4 has not been firmly established, with a wide range of responses observed from no effect (McFarlane et al. 2017; Terry et al. 2019b; Teoh et al. 2019) to a 25% reduction (Saleem et al. 2018). These differences in responses might be due to the variability in physical and chemical characteristics of the biochar products used in the studies. Hence, the evaluation of biochar effects using *in vitro* approaches can be a viable attempt to determine the intrinsic properties of biochar that can result in favorable outcomes in terms of CH_4 emissions.The experiments in this study assessed the effects of seven biochar products differing in source, level of inclusion and particle size on *in vitro* DMD, total gas and CH_4 production and rumen fermentation parameters of a barley silage-based diet typical of that fed to growing cattle in western Canada.

Materials and methods

Animal care and handling

The animals used in the study were handled in accordance to the Canadian Council on Animal Care guidelines (CCAC 2009), with experimental procedures approved by the University of Manitoba Animal Care Committee.

Basal experimental diet

The basal diet was composed of 600 barley silage, 270 dry-rolled barley grain, 100 canola meal and 30 mineral/vitamin supplement (g kg^{-1} DM basis). The supplement consisted of 565 barley grain, 250 calcium carbonate, 100 canola meal, 30 salt, 25 molasses, 10 premix and 0.66 vitamin E (g kg^{-1} DM basis). The premix was comprised of essential minerals: 65 Zinc, 28 Manganese, 15 Copper, 0.7 Iodine, 0.3 Selenium and 0.2 Cobalt (mg kg^{-1} DM basis). The supplement also contained vitamin A (6000 IU) and vitamin D (600 IU).

Inoculum source

Three ruminally-cannulated Aberdeen Angus heifers were used as rumen fluid donors for both experiments and were housed at the University of Manitoba Glenlea Research Station. Heifers were offered ad libitum access to a TMR diet (as per the basal experimental diet) and fed once daily (0800-0830h) for 14 d prior to rumen sampling in both experiments.

Treatments and experimental design

Two *in vitro* batch culture experiments were conducted to evaluate the effects of biochar as a feed additive in a barley silage-based TMR diet on DMD, total gas and CH_4 production and rumen fermentation parameters. Both experiments were conducted as randomized complete block designs with three laboratory replicates and three runs per experiment. The biochar products used in this study were supplied by Cool Planet® (Greenwood Village, CO, USA) and pyrolyzed from coconut husks (CP001, CP014) or pine (CP002, CP015, CP016, CP023, CP024). The carbon content, pH and particle size distribution of all biochars were determined (Table 1). The barley silage-based diet without biochar was included as a control in all experiments.

In Exp. 1, the seven biochar products listed above were assessed at three levels of inclusion (4.5, 13.5 and 22.5% diet DM) after a 24 h incubation period. In Exp. 2, CP002, CP016 and CP023 were selected to provide a range of physical (bulk density, surface area, pore volume, particle size distribution) and pHs across the available products. These products were assessed at two levels of inclusion (2.25 and 4.50% diet DM) and three particle sizes (< 0.5, 0.5-2.0, > 2.0 mm) over a 48 h incubation period. To achieve the desired particle size, the biochar products were ground using a

Wiley Mill (Thomas Scientific., Swedesboro, NJ, USA) with the corresponding sieve size of 0.1, 0.5 and 2.0 mm.

In vitro incubation

Both *in vitro* experiments were conducted in accordance to the procedures described by Menke et al. (1979). The diet and biochar products were oven-dried at 55°C for 48 h and thereafter, the diet was ground (Thomas Scientific., Swedesboro, NJ, USA) through a 1 mm screen. The diet was weighed (0.5 g DM) individually into incubation vials (capacity 120 mL) and the respective type and amount of biochar added for each incubation. Buffer mineral solution (McDougall 1948) was prepared and maintained at 39°C, and continually flushed with CO_2 prior to and during dispensing. Rumen fluid was collected 2 h post-feeding from donor heifers from four different locations in the rumen and strained through a Pecap mesh (mesh size 250 µm; PA66CG-250 136 cm, Sefar Nytal, Gilbert Saguenay, QC, CA). The fluid was then composited in pre-warmed thermoses and filtered through three layers of cheese cloth in the laboratory. Rumen fluid was transferred into a pre-heated 4 L glass jar maintained at 39°C in a water bath where CO_2 was continuously flushed to maintain anaerobic conditions. The inoculum, consisting of 15 mL rumen fluid and 30 mL buffer mineral solution, was dispensed into each vial and sealed with butyl rubber stoppers and aluminum crimp caps. The vials were then placed on orbital shakers (speed at 60 rpm, TYZD-III orbital shaker; Jiangsu Tenlin Instrument, Jiangyan, China) inside an incubator set at 39 °C (VWR Scientific, Model 2020, Mississauga, ON, CA) for 24 h and 48 h of incubation in Exp. 1 and 2, respectively.

Experimental Measurements

Dry matter disappearance

The DMD was determined (expressed as coefficient of digestibility) as the difference between the substrate DM (TMR with or without biochar) and dry weights of sample residues in the diet before and after incubation, divided by the substrate DM in the diet prior to incubation. Solid residues from each vial were transferred into pre-weighed falcon tubes (50 mL) and centrifuged at 1575 × g (Thermo Scientific, Sorvall Legend X1R, NH, USA) for 15 min at 4 °C. The resulting supernatant was discarded, after filtering through a mesh (mesh size 250 µm; PA66CG-250 136 cm, Sefar Nytal, Gilbert Saguenay, QC, CA) and the residue was retained.

Incubation vials were flushed with distilled water and centrifuged a total of three times to ensure that all solid contents were collected. After centrifugation, the final supernatant was filtered and discarded and the solid pellets were oven-dried for 48 h at 55 °C. These samples were weighed and the residue weight recorded to calculate DMD.

Total gas and CH_4 production

For Exp. 1, gas was collected at 3, 6, 9, 12 and 24 h, whereas for Exp. 2 gas additional collections were added at 18, 36 and 48 h. Gas pressure was recorded at all time-points using a pressure transducer (Traceable® pressure calibrator, model 33500-086, VWR international, Friendswood, TX, USA). A 10 mL sample of gas was collected at each timepoint from each vial via syringe (20 mL) and injected (25-gauge, ½ needle) into 6.8 mL exetainers (Labco, Ltd., Wycombe, London, UK). The gas pressure measurements obtained at each time point were totalled to calculate total gas production using the equation of Mauricio et al. (1999). Methane production was determined from gas samples via gas chromatography (GC; Agilent 7890B series GC custom, Agilent Technologies Canada Inc., Mississauga, ON, CA). The GC apparatus had an inlet temperature of 150 °C and a constant pressure of 30 psi, with a total septum purge flow of 3 mL/min. Gas samples were manually injected into the front inlet detector (250 °C; airflow rate of 450 mL min^{-1}; H_2 flow rate 70 mL min^{-1}). In both experiments, total gas and CH_4 production were expressed as cumulative values from all time points and were expressed as mL/g DMD and mL/g DM incubated.

Rumen fermentation parameters

Following incubation in each experiment, vials were immediately placed on ice and the pH of the liquid fractions recorded. For VFA analysis, 3 mL of the fermented fluid was collected from each vial into pre-filled tubes containing 25% metaphosphoric acid (0.6 mL) as per the techniques of Erwin et. al (1961) and stored at -20 °C until analyzed. For the analysis, VFA samples were thawed and mixed with 25% sodium hydroxide (0.240 mL) and 0.3 M oxalic acid (0.384 mL) and centrifuged at 1008 × g for 20 min (Thermo Scientific, Sorvall Legend X1R, NH, USA). The resulting supernatant (1 mL) was analyzed using GC (Varian 3900, Walnut Creek, CA, USA) equipped with an auto sampler (CP 8400, Walnut Creek, CA, USA). The total VFA concentrations

as well as molar proportions of acetate, propionate, butyrate, branched VFAs (BCVFA; isovalerate and isobutyrate) and the acetate to propionate (A: P) ratio were determined.

For NH_3-N analysis, 3 mL of fluid collected from each vial was mixed with 7.2 N sulfuric acid (0.6 mL) and the samples stored at -20 °C. Subsequently, samples were thawed and prepared via Indole-Phenol method (Novosamsky et al. 1974) and analyzed using a UV spectrophotometer (Ultrospec 3100 pro UV/Visible, Cambridge, England, UK). The NH_3-N concentrations were determined via measuring absorbance at 655 nm (Microplate Manager 4.0, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data were analyzed using PROC MIXED in SAS (SAS, 2018; university edition for Linux, SAS Institute, Inc., Cary, NC, USA). Both experiments were conducted as a randomized complete block design, with each treatment having three laboratory replicates and three runs per experiment. The rotary oscillators in the incubator described previously were designated the blocking effect as they sat at different levels in the incubator. In Exp. 1, the model parameters included the fixed effects of treatment (biochar product), level of inclusion and treatment × level of inclusion interaction, with run and replicate considered random effects. In Exp. 2, the fixed effects were treatment, level of inclusion, particle size and the interaction between these fixed effects namely treatment × level of inclusion, treatment × particle size, level of inclusion × particle size, treatment × level of inclusion × particle size. Both experiments had least square means calculated for DMD, total gas and CH_4 production, pH, VFA and NH_3-N and these values were subtracted from the control mean and expressed as deviations to allow for factorial analysis and comparisons with each replicate at specific levels of inclusion. Orthogonal polynomial contrasts were performed to test parameter responses (linear, cubic, quadratic) to biochar inclusion and to directly compare each biochar treatment with the control. The type I error rate for hypothesis testing was 0.05 (P values < 0.05 were considered significant).

Results

Dry matter disappearance

In Exp. 1, biochar affected DMD ($P = 0.003$; Table 2), with the CP001 significantly lower than the control, and a numerical but non-significant reduction in DMD with CP014 (Table 3).

Furthermore, a negative linear response ($P = 0.002$) was observed with increasing level of biochar inclusion (Table 4). However, no biochar × level of inclusion interactions were observed ($P = 0.554$). In Exp. 2, biochar inclusion did not affect DMD regardless of biochar product ($P = 0.336$), level of inclusion ($P = 0.232$) or particle size ($P = 0.720$), with no interactions ($P > 0.05$) observed (Table 5). In Exp 2, physical (surface area, pore and particle size, bulk density) and chemical (C, and ash content as well as pH) did not elicit apparent differences in DMD.

Total gas and CH_4 production

In Exp. 1, relative to the control, biochar product did not affect ($P \geq 0.101$) total gas production expressed in mL/g DM incubated or mL/g DMD (Table 2; Table 3). Level of inclusion also did not affect total gas production ($P \geq 0.559$) or result in linear or quadratic responses ($P \geq 0.288$; Table 4). Additionally, there were no biochar × level of inclusion effects ($P \geq 0.101$; Table 2) on total gas production irrespective of unit of expression. In Exp. 2, compared to the control, biochar did not affect ($P \geq 0.222$) total gas production (Table 5; Table 6) and any of the inclusion levels ($P \geq 0.221$; Table 7). Particle size also did not affect total gas production ($P \geq 0.151$), with no interactions observed ($P \geq 0.381$; Table 5).

In Exp. 1, CH_4, expressed in mL/g DM incubated and mL/g DMD, was not affected by biochar regardless of treatment ($P \geq 0.398$; Table 2; Table 3). Level of inclusion did not affect ($P \geq 0.246$) total CH_4 produced ($P \geq 0.137$; Table 4). There were also no treatment × level of inclusion interactions for CH_4 ($P \geq 0.286$; Table 2). Similarly, in Exp. 2, biochar regardless of product, did not affect ($P \geq 0.369$) CH_4 production irrespective of how it was expressed (Table 5; Table 6). Level of inclusion ($P \geq 0.567$) and biochar particle size ($P \geq 0.367$) did not affect CH_4 production (Table 7), with no linear or quadratic responses ($P \geq 0.254$; Table 8) and no interactions ($P \geq 0.073$; Table 5). In both experiments, physical (surface area, pore and particle size, bulk density) and chemical (C, and ash content, pH) did not elicit any responses in associated gas parameters as evidenced by the results.

Rumen fermentation parameters

In Exp. 1, biochar did not affect rumen pH ($P = 0.236$; Table 3) or level of inclusion did not effect ($P = 0.305$; Table 4) rumen pH or result in interactions ($P = 0.697$: Table 2). Similarly,

in Exp. 2 neither biochar product, inclusion level (Table 7) or particle size (Table 8) affected pH (P = 0.629) compared to the control.

In Exp. 1, total VFA concentration was not affected by biochar product, inclusion level and no interactions were observed ($P \geq 0.174$; Table 2). Additionally, neither individual VFA concentrations (acetate, butyrate and propionate) nor BCVFA or A:P ratios were affected ($P \geq 0.104$; Table 3), with similar results observed in Exp. 2 (Table 5). Individual VFA concentrations, as well as BCVFAs and A:P ratios were not affected ($P \geq 0.107$; Table 6) by biochar or level of inclusion with no interactions. Particle size of biochar had no effect on total and individual VFA concentrations, nor the BCVFAs and A:P ratios ($P \geq 0.175$; Table 8).

In Exp. 1, the concentrations of NH_3-N (mg/dL) were unaffected by biochar (P = 0.168; Table 2) relative to the control. Level of inclusion did not affect NH_3-N (P = 0.223) and no linear, quadratic ($P \geq 0.101$) or treatment × level of inclusion responses were observed (P = 0.061). In Exp. 2, biochar treatment did not affect (P = 0.679; Table 5) NH_3-N concentrations compared to the control. Furthermore, results show that level of inclusion did not affect NH_3-N (P = 0.142; Table 7) and there was no particle size effect (P = 0.719; Table 8), with no interactions. Rumen fermentation parameters, in both experiments, was not affected by biochar regardless of physical (surface area, pore and particle size, bulk density) and chemical (C, and ash content, pH) characteristics.

Discussion

Dry matter disappearance

In the current *in vitro* study, biochar treatment and level of inclusion decreased DMD in Exp. 1, whereas no significant response was observed on Exp. 2. The biochar products in Exp. 1 were included at (4.5, 13.5 and 22.5 % diet DM, (0.5, 1.5, and 2.5 mg/ml of vial inoculum, respectively) which were higher than the levels of inclusion in Exp. 2 (2.25 and 4.5 % diet DM;0.25 and 0.5 mg/ml of inoculum respectively) which may explain the different responses observed. Two of the levels in Exp. 1 were also higher than that in the study of Hansen et al. (2012) who reported numerical reductions (2-12%) in DMD of biochar supplemented hay mixed ration *in vitro* evaluating the effects of wood-based and activated biochar included at 9 % of diet DM. In Exp. 1, DMD was reduced significantly with the coconut biochar; which may be attributed to it and other endocarp containing fruits (olives, walnuts) having the highest lignin content of all plant tissues

(Welker et al. 2015). This may have resulted in the negative impact on DMD compared to the pine-based treatments. Lignin may impact fiber digestibility as its polymers may form cross-linkages with cellulose and hemi-cellulose with covalent bonds, which results in less digestible carbohydrates (Srivastava et al. 2012; Tarasov et al 2018). The high level of biochar inclusion, in addition to its indigestible lignified composition, may have impeded colonization of fibrolytic and cellulolytic bacteria that are involved in feed digestion (Schmidt and Noack 2000). However, McFarlane et al. (2017) reported no significant responses in forage DMD of orchard grass hay when 8.1 % of the DM was composed of wood-based biochars (yellow poplar, white pine, chestnut oak) *in vitro*. Notwithstanding, the lack of DMD response in Exp. 2 also agreed with the Teoh et al. (2019) where the DMD of oaten pasture/maize silage/concentrate TMR was not affected by biochar inclusion. Saleem et al. (2018) observed a significant linear increase in DMD of a barley silage-based TMR in an *in vitro* RUSITEC system with increasing, but lower levels (0.5, 1.0, 2.0 % diet DM) of CP016 than the current experiments. These authors speculated that this positive response resulted from the porous nature of biochar, which is reported to aid in housing surrounding microbiota which can result in improved biofilm formation (Leng et al. 2013). With the increased cellulolytic and fibrolytic bacteria, the degradation of substrate may be more efficient. Thus, in the current study, effects of pore volume and surface area of biochars would have been expected, with biochars having high pore volumes and surface areas eliciting positive responses in both DMD and associated gas parameters due to enhanced biofilm formation; however, this was not the case. Lastly, particle size was not observed to affect DMD which was in agreement with McFarlane et al. (2017) where biochar did not affect forage digestibility, as DMD in control samples were similar to biochar treatments; however, these authors observed significantly higher DMD in samples supplemented with smaller particle size biochar (< 0.178 mm) compared to larger particle size biochar (> 0.178 mm). This suggests that particle size influenced rumen kinetics as smaller sized compared to larger sized biochar may have less adverse impacts on digestibility, owing to increasing surface area which was in agreement with increased gas production in their *in vitro* study. The increased surface area may also be more favorable for microbial colonization and thus resulted in improved DMD in their study. However, particle size effects were not apparent in Exp. 2 with no commensurate responses were observed in either DMD or total gas production. Moreover, the correlation of particle size distribution to biochar particle size is unclear as the particle size distribution may been altered upon processing the biochar

products into smaller particle sizes prior to the *in vitro* study. The C content of the biochars ranging from 71.1 to 81.6 % DM which were similar, hence the lack of differences in DMD. This was in agreement with other biochar studies having used lower (C 10.0 % DM; Teoh et al. 2019) or higher (C 91.2 % DM; Hansen et al. 2012) C content having no effects on DMD. The biochar pHs ranged from slight acidic to neutral (4.9 to 7.3) and also did not have an effect on DMD, similar to the results of Hansen et al. (2012) and Teoh et al. (2019) who used alkaline biochars (pH 9.6 and 8.2, respectively).It is reported that biochar, especially alkalinic types, may exhibit buffering capacity in anaerobic digestion of complex organic wastes by acting as a termporary electron acceptor and promoting the oxidation of butyrate, preventing decreases in pH (Wang et al. 2018); however this was not the case in the current study and the cited literature.

Total gas and CH_4 production

In both experiments, total gas and CH_4 production were not affected by biochar inclusion regardless of treatment, level of inclusion or particle size which is in agreement with Cabeza et al. (2018) who observed no effect on in vitro total gas or CH_4 production with a hay-based diet regardless of biomass source (Miscanthus straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw). Moreover, McFarlane et al. (2017) reported no differences in total gas production among biochar samples regardless of biomass source but observed higher levels in samples supplemented with finer (< 0.178 mm) particle sized biochar, which aligned with an increase in DMD. Pereira et al. (2014) also observed no differences in total gas production between ryegrass ensiled with four biochars (pine wood chips and corn stover pyrolyzed at 350 vs 550°C) compared to control at three levels of inclusion (0.5, 1.0 and 2.0 % diet DM). However, biochar pyrolyzed at a lower temperature had higher total gas production compared to the that pyrolyzed at a higher temperature. In terms of CH_4 production, these authors did not observe any difference between the biochar and control diets. Additionally, Teoh et al. (2019) used a hardwood-based biochar (pyrolyzed at 650°C) in a RUSITEC with silage and observed no significant reductions in CH_4 production. In an *in vivo* study, Terry et al. (2019b) also found that addition of a pine-based biochar (0, 0.5, 1 and 2 % diet DM) to a barley-silage based diet did not alter CH_4 production in beef heifers. Collectively, the lack of a response in total gas and CH_4 production with the addition of biochar to the diet agrees with the current study. However, in contrast, Saleem et al. (2018) reported a significant reduction in CH_4 production (7.7-22.0 %, depending on unit of expression),

with a concurrent linear increase in DMD with CP016 included in a silage-based diet in an artificial rumen system. The differences in the results of Saleem et al. (2018) and the current study are not fully understood and may be attributed to the experimental model (batch culture vs RUSITEC), biochar products, as well as levels of inclusion. Furthermore, in an *in vivo* study, Leng et al. (2012c) reported a 24.3% reduction of CH_4 with 0.6% diet DM inclusion of a rice hull-based biochar pyrolyzed at 900°C to south east Asian cattle fed cassava roots and foliage. In that study, cattle feed intake was unaffected by biochar inclusion, hence the reduction may be partially attributed to the hypothesis that biochar aids in the formation of methanotrophic consortia on the inert surfaces on the biochar (Leng et al. 2014; Kammann et al. 2017). However, this hypothesis was not confirmed and maybe unlikely as methanotrophs are often absent or present in extremely low numbers in the rumen (Henderson et al. 2015; Parmar et al. 2015; Auffret et al. 2018). Biochar is also hypothesized to facilitate redox reactions between syntrophic (fermentative bacteria and methanogens) microbial populations resulting in improved microbial growth (Leng et al. 2013; Kammann et al. 2017). The enhanced microbial growth is suggested to encourage biofilm formation and improve feed conversion efficiency, therefore decreasing CH_4 output per unit of feed (Leng et al. 2012a, b, c; Wu et al. 2016; Kammann et al. 2017). Notwithstanding, this may be unlikely as it is difficult to assess how a relative change in porosity would have an effect on the microbial activity and as differing pore volumes, surface areas and particle sizes in biochar elicited no effect on measured parameters in this study. Moreover, biofilm formation and microbial colonization may not be as well developed in biochar surfaces as compared to feed particles in the rumen. This was further supported by electron microscopy analysis completed by Terry et al. (2019b) comparing feed and biochar surface biofilms, and the metagenomic data analysis of rumen microbiota in the biochar studies of Teoh et al. (2019) and Terry et al. (2019b). These authors observed no overall significant improvements in rumen fermentation regardless of shifts in select rumen microbial groups.

Several *in vitro* studies (Pereira et al. 2014; McFarlane et al. 2017; Cabeza et al. 2018; Teoh et al. 2019), report that biochar product, regardless of source/chemical/physical composition did not mitigate rumen CH_4 emissions. It is well established that the disappearance of feed substrate in the rumen (and resultant VFA patterns) influences CH_4 production (Johnson and Johnson 1995; Winders et al. 2019). This is in agreement with the results of Exp. 2, with the absence of responses in DMD, total gas/CH_4 production or associated fermentation parameters.

Notwithstanding, although a significant decrease in DMD was observed in Exp. 1 no decrease in total gas or CH_4 production was identified. This reduction in DMD was small (3.0 %) and thus might not have been biologically meaningful enough result in a reduction in in gas production. Similarly, Hansen et al. (2012) reported no change in total gas production (mL/g DM) *in vitro* when biochar (9 % diet DM) was included in a hay-based TMR diet, which corresponded to no significant change in DMD. In terms of particle size, differently sized biochar in Exp. 2 did not affect gas or CH_4 production.

Pyrolysis temperature can influence the overall physiochemical profile of biochar and its consequent effect on ruminal microbiota as observed in the reports of Leng et al. (2012a, b, c; 2013). The current experiments used biochars that were pyrolyzed at temperatures ranging from 400 to 600 °C which was similar to Peirera et al. 2014 (350 and 550 °C) and Teoh et al. 2019 (650 °C) but lower than Leng et al. (2012a, b, c) who utilized biochars pyrolzed at 900 °C. High pyrolysis temperatures (600 – 1000 °C) are reported to increase pore volumes, thereby increasing the surface area of biochar as large amounts of volatile substances escape the cellular structure (Rizkiana et al. 2014). This favors the formation of internal pores within biochar particles (Paethanom and Yoshikawa 2012; Rizkiana et al. 2014) and transforms it to a more amorphous graphene like form (Kalachniuk 1994). The increased porosity can provide microhabitats and propagate populations (lignocellulosic microbiota) in the biofilm and the graphene structure of high temperature pyrolyzed biochar is hypothesized to mediate electron swapping between microbial groups in metabolic processes more easily as described previously (Leng et al. 2013; Leng 2014). Nevertheless, it is still unclear how biochar with increased porosity included in diets at relatively low levels can impact the ruminal microbiota and fermentation given the reports on microbial colonization and metagenomic analyses on biochar studies. Digestible sugars and available carbohydrates are also likely combusted in the pyrolysis step in biochar production, which likely renders biochar its indigestible and inert nature hence being unmetabolizable in the rumen. Another factor that may have contributed to the decreased CH_4 production in the study of Leng et al. (2013) is that they have used a non-common tannin-rich diet (cassava root and foliage) which may have served as an alternative terminal acceptor affecting methanogenesis (Schmidt et al. 2019).

Rumen fermentation parameters

The rumen fermenter fluid pH was not affected by the differing biochar products in either experiments and reflected the commonality of VFA concentrations across incubations. The absence of an effect on VFAs was in agreement with Pereira et al. (2014) also reported that molar proportions of VFAs were unaffected by biochar *in vitro*, regardless of source (pine wood or straw-based) or level of inclusion (0. 2.1, 4.2, 8.1, 18.6 % diet DM) to hay silage-based diet. This was further confirmed by McFarlane et al. (2017) who observed no effect of biochar source (chestnut oak, yellow poplar and white pine) when included at 8.1 % of diet DM in an orchard grass-based diet, regardless of particle size (<0.178 or > 0.178 mm). Cabeza et al. (2018) also observed an overall lack of response in total VFA in a hay-based batch culture experiment but observed a decrease in propionate and butyrate in biochar treatments. However, the decrease in molar proportions of these VFAs might have not been biologically meaningful as there were no changes in total gas or CH_4 production.

Biochar's effect on NH_3-N production is not definitive as studies have reported a range from no effect (Pereira et al. 2014) to both a decrease (Cabeza et al. 2018) and an increase (Saleem et al. 2018). The latter authors suggested that this increase may be due to increased ruminal protein degradation, which was also evidenced by increased branched VFA concentrations in their study. Ruminal NH_3-N concentration is an indicator of the efficiency of conversion of dietary N to microbial N (Firkins et al. 2007) and it is suggested that biochar can enhance AA deamination (Saleem et al. 2018). Cabeza et al. (2018), who observed a decrease in NH_3-N, suggested that biochar (1.2 -12 % diet DM) may have adsorbed the NH_3-N, a phenomenon also reported as biochar prevents NH_3-N leaching in soils (Ding et al. 2010). This is not clear, however, as biochar, being enriched with recalcitrant carbon may have different effects on soils as compared to the rumen. Biochar amendment may be advantageous in soils as C:N ratios are improved, with NH_3 volatilization being regulated via absorbing NH_4^+, resulting in decrease in N_2O emissions and N leaching (Kamman et al. 2017). This might not be the case in the rumen as the residence time is much shorter and the synchronization of C and N is unlikely due to biochar's recalcitrance. Therefore, this in addition to the lack of response to DMD and VFA results, may have resulted in the concomitant response in NH_3-N.

Conclusions

The inclusion of biochar products differing in source, physical and chemical characteristics, as well as particle size did not affect DMD, total gas or CH_4 production or rumen fermentation in a barley silage-based TMR. Thus, in this i*n vitro* study, biochar as a feed additive did not demonstrate potential to mitigate enteric CH_4 in barley silage-based diets.

Table 1. Source, chemical and physical characteristics of seven biochar products[z] used in in vitro Exp 1 and 2.

	Biochar product						
Parameter	CP001	CP002	CP014	CP015	CP016	CP023	CP024
Source/biomass origin	Coconut	Pine	Coconut	Pine	Pine	Pine	Pine
Chemical characteristics							
Carbon, % DM	75.6	81.6	76.6	75.4	76.9	75.3	71.1
pH	6.3	5.8	5.0	4.9	4.9	7.6	7.3
Physical characteristics							
Bulk density, kg/m^3	706.0	310.0	606.0	262.0	287.0	122.0	140.0
Surface area, m^2/g	161.0	218.0	160.0	189.0	186.0	152.0	148.0
Pore volume, cc/g	6.45×10^{-2}	8.75×10^{-2}	6.52×10^{-2}	7.56×10^{-2}	7.36×10^{-2}	6.10×10^{-2}	6.00×10^{-2}
Particle size distribution[y], mm							
D 0.1	0.25	0.15	0.29	0.29	1.60	0.85	0.73
D 0.5	0.95	1.25	0.47	0.50	4.30	1.95	1.75
D 0.9	1.85	2.95	0.73	0.83	5.95	3.00	3.15

[z] measurements on biochar products' physical and chemical characteristics provided by Cool Planet®

[y] particle size distribution: where "D" is the diameter and the number (0.1, 0.5, 0.9) are probabilities of finding a particle size (undersized, average size, oversized in the batch) with the diameter D in a given volume; with D0.5 being the median particle size.

Table 2. Significance (*P* values) of biochar treatment (product) and level of inclusion in a TMR-based diet[z] on measured variables in Exp 1.

			Response		Interaction
Parameter	Treatment	Level of inclusion	Linear	Quadratic	Treatment × Level of inclusion
DMD	0.003	0.001	0.002	0.074	0.554
Total gas production, mL/g DM incubated	0.848	0.907	0.974	0.663	0.195
Total gas production, mL/g DMD	0.101	0.559	0.288	0.932	0.101
CH_4, mL/g DM incubated	0.889	0.246	0.450	0.137	0.640
CH_4, mL/g DMD	0.398	0.369	0.223	0.483	0.286
pH	0.236	0.305	0.204	0.816	0.697
Total VFA, mmol/L	0.223	0.300	0.689	0.138	0.174
Acetate	0.476	0.253	0.354	0.167	0.377
Propionate	0.119	0.287	0.121	0.823	0.765
Butyrate	0.656	0.270	0.136	0.574	0.662
BCVFAs[y]	0.798	0.211	0.104	0.556	0.830
A:P[x]	0.435	0.377	0.202	0.568	0.589
NH_3-N, mg/dL	0.168	0.223	0.597	0.101	0.061

[z] Biochar products were included in TMR at three levels of inclusion (4.5, 13.5 and 22.5 % diet DM)

[y] Isovalerate + Isobutyrate

[x] Acetate to propionate ratio

Table 3. Effects of biochar treatment (product) on DMD, total gas and CH_4 production and rumen fermentation parameters[z] of control and biochar treatments[y] in Exp 1.

		Biochar treatment								
Parameter	Control	CP001	CP002	CP014	CP015	CP016	CP023	CP024	SEM	*P* value
DMD	0.52b	0.50a	0.52b	0.51ab	0.52b	0.52b	0.52b	0.52b	0.004	0.003
Total gas production, mL/g DM incubated	159.80	160.84	157.99	158.73	157.12	158.27	157.36	158.08	1.800	0.848
Total gas production, mL/g DMD	161.90	164.30	159.51	162.23	160.59	160.75	161.69	164.59	2.505	0.101
CH_4, mL/g DM incubated	52.70	53.02	51.66	51.52	51.71	51.08	52.24	52.76	0.796	0.889
CH_4, mL/g DMD	53.75	53.40	52.73	51.61	51.99	51.91	52.45	53.63	0.861	0.398
pH	6.32	6.30	6.31	6.31	6.32	6.29	6.31	6.32	0.023	0.236
Total VFA, mmol/L	75.01	70.40	72.50	80.73	75.21	78.92	67.68	79.98	12.352	0.223
Acetate	45.16	41.43	42.67	48.25	44.20	47.87	40.49	47.82	9.143	0.476
Propionate	16.52	16.21	16.68	18.30	16.84	17.69	14.38	18.23	2.215	0.119
Butyrate	10.45	10.05	10.47	11.30	11.50	10.64	10.34	11.23	2.070	0.656
BCVFAs[x]	2.88	2.71	2.68	2.88	2.67	2.72	2.47	2.70	0.543	0.798
A: P[w]	2.75	2.79	2.82	2.57	2.63	2.74	2.77	2.72	0.342	0.435
NH_3-N, mg/dL	11.70	10.10	9.77	10.73	10.79	10.43	10.58	10.17	0.294	0.168

[z] Least square means were expressed as the sum of TMR parameter value observed and deviations from TMR value added.

[y] Biochar products were included in TMR at three levels of inclusion (4.5, 13.5 and 22.5 % diet DM)

[x] Isovalerate + Isobutyrate

[w] Acetate to propionate ratio

Table 4. Effects of biochar level of inclusion on DMD, total gas and CH_4 production and rumen fermentation parameters[z] of control and biochar treatments in Exp 1.

		Level of inclusion					Response	
		% diet DM						
Parameter	Control (0)	4.5	13.5	22.5	SEM	*P* value	Linear	Quadratic
DMD	0.52a	0.52a	0.52a	0.50b	0.002	0.001	0.002	0.074
Total gas production, mL/g DM incubated	159.80	158.48	157.84	158.53	1.044	0.907	0.974	0.663
Total gas production, mL/g DMD	161.90	161.60	163.09	163.21	2.478	0.559	0.288	0.932
CH_4, mL/g DM incubated	52.70	51.69	53.07	52.31	0.464	0.246	0.450	0.137
CH_4, mL/g DMD	51.75	51.75	52.54	52.54	0.503	0.369	0.223	0.483
pH	6.32	6.32	6.30	6.30	0.023	0.305	0.204	0.816
Total VFA, mmol/L	75.01	72.03	77.86	75.63	11.465	0.300	0.689	0.138
Acetate	45.16	41.88	47.25	44.89	8.713	0.253	0.354	0.167
Propionate	16.52	16.18	16.88	18.00	2.020	0.287	0.121	0.823
Butyrate	10.45	11.17	10.99	10.21	1.993	0.270	0.136	0.574
BCVFAs[y]	2.88	2.80	2.74	2.53	0.526	0.211	0.104	0.556
A: P[x]	2.75	2.79	2.69	2.72	0.342	0.377	0.202	0.568
NH_3-N, mg/dL	11.70	10.31	10.63	10.17	0.902	0.223	0.597	0.101

[z] Least square means were expressed as the sum of TMR parameter value observed and deviations from TMR value added.

[y] Isovalerate + Isobutyrate

[x] Acetate to propionate ratio

Table 5. Significance (*P* values) of biochar treatment (product), level of inclusion and particle size in a TMR-based diet[z] on measured variables in Exp 2.

	Response						Interaction			
Parameter	Treatment (T)	Level of inclusion (L)	Particle size (S)	L, Linear	S, Linear	S, quadratic	T × L	T × S	L × S	T × L × S
DMD	0.336	0.232	0.720	0.232	0.440	0.819	0.881	0.700	0.486	0.202
Total gas production, mL/g DM incubated	0.565	0.588	0.490	0.588	0.793	0.254	0.456	0.805	0.381	0.766
Total gas production, mL/g DMD	0.222	0.221	0.151	0.221	0.229	0.120	0.461	0.364	0.820	0.900
CH_4, mL/g DM incubated	0.962	0.590	0.367	0.590	0.254	0.403	0.073	0.458	0.703	0.660
CH_4, mL/g DMD	0.369	0.567	0.388	0.567	0.372	0.293	0.208	0.283	0.588	0.698
pH	0.629	0.424	0.896	0.424	0.749	0.739	0.865	0.874	0.616	0.967
Total VFA, mmol/L	0.838	0.879	0.573	0.879	0.752	0.318	0.774	0.897	0.341	0.150
Acetate	0.522	0.323	0.228	0.323	0.810	0.824	0.862	0.721	0.475	0.281
Propionate	0.172	0.997	0.667	0.997	0.464	0.598	0.794	0.736	0.390	0.883
Butyrate	0.107	0.528	0.654	0.528	0.391	0.736	0.391	0.349	0.735	0.709
BCVFAs[y]	0.354	0.610	0.283	0.610	0.212	0.319	0.742	0.824	0.285	0.796
A: P[x]	0.287	0.305	0.175	0.305	0.640	0.998	0.864	0.772	0.290	0.275
NH_3-N concentration, mg/dL	0.679	0.142	0.719	0.142	0.453	0.768	0.889	0.370	0.691	0.931

[z] Biochar products were included in TMR at two levels of inclusion (2.3, 4.5 % diet DM) and three particle sizes (< 0.5, 0.5-2, > 2 mm)

[y] Isovalerate + Isobutyrate

[x] Acetate to propionate ratio

Table 6. Effects of biochar treatment (product) on DMD, total gas and CH_4 production and rumen fermentation parameters[z] of control and biochar treatments[y] in Exp 2.

		Biochar treatment				
Parameter	Control	CP002	CP016	CP023	SEM	*P* value
DMD	0.55	0.55	0.56	0.56	0.002	0.336
Total gas production, mL/g DM incubated	177.73	178.04	176.47	175.45	6.038	0.565
Total gas production, mL/g DMD	194.82	192.73	193.63	190.21	2.498	0.222
CH_4, mL/g DM incubated	65.02	65.21	64.37	64.34	1.323	0.369
CH_4, mL/g DMD	71.39	72.37	71.43	71.94	1.214	0.962
pH	6.04	6.05	6.05	6.05	0.010	0.629
Total VFA, mmol/L	121.47	123.89	121.24	120.28	8.581	0.838
Acetate	83.65	86.03	83.73	84.21	4.009	0.522
Propionate	23.32	23.43	23.08	22.35	0.809	0.172
Butyrate	14.50	14.56	14.43	13.72	0.518	0.107
BCVFAs[x]	6.48	6.35	6.23	6.26	0.263	0.354
A: P[w]	3.59	3.75	3.65	3.74	0.096	0.287
NH_3-N, mg/dL	38.09	39.01	38.65	38.34	1.883	0.679

[z] Least square means were expressed as the sum of TMR parameter value observed and deviations from TMR value added.

[y] Biochar products were included in TMR at two levels of inclusion (2.3, 4.5 % diet DM) and three particle sizes (< 0.5, 0.5-2, > 2 mm)

[x] Isovalerate + Isobutyrate

[w] Acetate to propionate ratio

Table 7. Effects of biochar level of inclusion on DMD, total gas and CH_4 production and rumen fermentation parameters[z] of control and biochar treatments[y] in Exp 2.

	Level of inclusion % diet DM				
Parameter	Control (0)	2.3	4.5	SEM	*P* value
DMD	0.55	0.55	0.56	0.002	0.232
Total gas production, mL/g DM incubated	177.73	176.11	177.20	5.957	0.588
Total gas production, mL/g DMD	194.82	191.03	192.33	2.354	0.221
CH_4, mL/g DM incubated	65.02	64.48	64.80	1.295	0.567
CH_4, mL/g DMD	71.39	71.68	72.05	1.209	0.590
pH	6.04	6.05	6.05	0.010	0.424
Total VFA, mmol/L	121.47	122.6	121.21	8.174	0.879
Acetate	83.65	85.51	83.80	3.922	0.323
Propionate	23.32	22.96	22.96	0.771	0.997
Butyrate	14.50	14.13	14.45	0.485	0.528
BCVFAs[x]	6.48	6.22	6.27	0.267	0.610
A: P[w]	3.59	3.74	3.68	0.081	0.305
NH_3-N concentration, mg/dL	38.09	38.20	39.13	1.858	0.142

[z] Least square means were expressed as the sum of TMR parameter value observed and deviations from TMR value added.

[y] Biochar products were included in TMR at three particle sizes (< 0.5, 0.5-2, > 2 mm)

[x] Isovalerate + Isobutyrate

[w] Acetate to propionate ratio

Table 8. Effects of biochar particle size on measured DMD, total gas and CH_4 production and rumen fermentation parameters[z] of control and biochar treatments[y] in Exp 2.

		Particle size					Response	
		mm						
Parameter	Control	< 0.5	0.5-2	> 2	SEM	*P* value	Linear	Quadratic
DMD	0.550	0.560	0.560	0.560	0.002	0.720	0.440	0.819
Total gas production, mL/g DM incubated	177.73	177.15	175.03	178.30	6.038	0.490	0.793	0.254
Total gas production, mL/g DMD	194.82	191.89	190.34	194.33	2.499	0.151	0.229	0.120
CH_4, mL/g DM incubated	65.02	64.55	64.22	65.16	1.323	0.388	0.372	0.293
CH_4, mL/g DMD	71.39	71.59	71.47	72.48	1.219	0.367	0.254	0.403
pH	6.04	6.05	6.05	6.05	0.010	0.896	0.749	0.739
Total VFA, mmol/L	121.47	119.87	121.33	124.35	8.581	0.573	0.752	0.318
Acetate	83.65	82.95	84.39	86.63	4.009	0.228	0.810	0.824
Propionate	23.32	22.83	22.78	23.26	0.809	0.667	0.464	0.598
Butyrate	14.50	14.09	14.16	14.46	0.513	0.654	0.391	0.736
BCVFAs[x]	6.48	6.20	6.17	6.36	0.275	0.283	0.212	0.319
A: P[w]	3.59	3.64	3.71	3.72	0.089	0.175	0.640	0.998
NH_3-N, mg/dL	38.09	38.31	38.80	38.89	1.883	0.719	0.453	0.768

[z] Least square means were expressed as the sum of TMR parameter value observed and deviations from TMR value added.

[y] Biochar products were included in TMR at two levels of inclusion (2.3, 4.5 % diet DM)

[x] Isovalerate + Isobutyrate

[w] Acetate to propionate ratio

CHAPTER 2

Effect of pine-based biochars with differing physiochemical properties on methane production, ruminal fermentation and rumen microbiota in an artificial rumen (RUSITEC) fed barley silage

Abstract

This study investigated the effects of three pine-based biochar products on nutrient disappearance, total gas and CH_4 production, rumen fermentation, microbial protein synthesis and rumen microbiota in a rumen simulation technique (RUSITEC) fed a barley silage-based total mixed ration (TMR; 600 barley silage, 270 barley grain, 100 canola meal and 30 mineral/vitamin supplement g kg^{-1} on DM basis). Biochars, designated CP016, CP024 and CP028, were evaluated with each included at 20 g kg^{-1} of diet DM. A control treatment consisting of TMR (10 g) as per basal diet was also included. Biochars differed in bulk density, surface area, pore volume, pH and chemical composition (carbon and ash content). Treatments were assigned to 16 fermenters (n = 4 per treatment) in two RUSITEC units in a randomized block design. The experimental period was 17 d, with 10 d for adaptation followed by 7 d sampling and data collection. Data were analyzed using PROC MIXED in SAS, with treatment and day of sampling as fixed effects and RUSITEC unit and fermenters as random effects. Biochar did not affect disappearance of DM (P = 0.63), OM (P = 0.34), CP (P = 0.65), NDF (P = 0.12), ADF (P = 0.25) or starch (P = 0.38). Compared to the control, biochar did not affect total gas (P = 0.98) or CH_4 expressed as mg g^{-1} DM incubated (P = 0.48), mg g^{-1} DM disappeared (P = 0.27) or mg d^{-1} (P = 0.27). The total VFA (P = 0.65), NH_3-N production (P = 0.99) and total protozoa counts (P = 0.72) were not affected by biochar inclusion (P > 0.05). Microbial protein synthesis was not affected by biochar inclusion (P > 0.05). Moreover, alpha and beta diversity as well as rumen microbiota families were unaffected by biochar (P > 0.05). In conclusion, biochar did not reduce CH_4 emissions and did not affect nutrient disappearance, rumen fermentation, microbial protein synthesis or rumen microbiota in the RUSITEC.

Introduction

It is well established that ruminant livestock contribute to GHG, with 40% of livestock GHG emissions attributed to ruminal fermentation and slurries created from animal manure (FAO 2017). In Canada, the agriculture sector accounts for approximately 8.5% of the national total CH_4 anthropogenic emissions (Environment and Climate Change Canada 2020), of which 3 % is from rumen fermentation and livestock manures (Environment and Climate Change Canada 2017). Methane and CO_2 are the main gaseous by-products of ruminal fermentation, with enteric CH_4 accounting for 2-12% GEI with diet as a major factor accounting for this variability (Johnson and

Johnson 1995). As a result, there has been significant interest in using novel feed additives to potentially reduce enteric CH_4 emissions from ruminants. Furthermore, it has been reported that the inclusion of biochar in ruminant diets may offer CH_4 mitigation benefits (Leng 2014; Saleem et al. 2018).

Biochar is a carbon-rich product sourced from the pyrolysis of a variety of products such as animal wastes, as well as agricultural and lignocellulosic plant by-products (Lehmann and Joseph 2009). Biochar has been principally used as a soil amendment and reportedly adsorbs GHGs, as well as binds toxins and heavy metals (Joseph et al. 2015a, b). Additionally, Kammann et al. (2017) reported that the porous nature of biochar may benefit rumen bacteria (methanotrophs and propionate-producing bacteria) as it promotes the formation of microhabitats and improve microbial growth. It has been theorized that these properties will improve animal performance while decreasing CH_4 emissions (Lehmann and Joseph 2009; Feng et al. 2012; Leng 2014; Joseph et al. 2015a, b). Pyrolysis conditions during biochar production influence the pore size, surface area and carbon content of biochar. For example, biochar pyrolyzed at lower temperature tends to be more amorphous, with polar aliphatic groups compared to the graphene-like structure of biochar pyrolyzed at higher temperatures (Chen et al. 2008; Leng 2014; Kammann et al. 2017). It has been suggested that these differences affect the composition and activity of both soil and intestinal microbiota (Joseph et al. 2015a). In soils, biochar pyrolyzed at higher temperatures enhanced interactions among soil microbiota through increased interspecies electron transfer and the promotion of redox reactions (Mitsumori et al. 2012; Kammann et al. 2017). These redox reactions, along with the possibility of promoting methanotroph populations, have been proposed to reduce the intensity of CH_4 emissions in soils (Leng 2014).

However, there is no consensus regarding the efficiency of biochar as an additive in animal feeding systems, as CH_4 responses to biochar have ranged from no effect (Hansen et al. 2012; McFarlane et al. 2017; Terry et al. 2019b; Teoh et al. 2019) to a 25 % reduction (Saleem et al. 2018) *in vitro*. Therefore, this study assessed the effect of three biochar products, differing in physiochemical characteristics, on nutrient disappearance, total gas and CH_4 production, rumen fermentation, microbial protein synthesis and rumen microbiota in a rumen simulation technique (RUSITEC) fed a barley silage-based diet.

Materials and Methods

Animal care and handling

The animals used in the study were handled in accordance to the Canadian Council on Animal Care (CCAC 2009). Animal handling and sampling protocols employed were reviewed and approved by Agriculture and Agri-Food Canada Lethbridge Research and Development Centre Animal Care Committee.

Basal experimental diet

The basal experimental diet was a total mixed ration (TMR) that consisted of (g kg^{-1}, DM basis) 600 barley silage, 270 dry rolled barley grain, 100 canola meal and 30 mineral/vitamin supplement. The supplement was composed (g kg^{-1}, DM basis) of 565 barley grain, 250 calcium carbonate, 100 canola meal, 30 salt, 25 molasses, 10 premix and 0.66 vitamin E. The premix was composed of (mg kg^{-1} DM basis) 65 zinc, 28 manganese, 15 copper, 0.7 iodine, 0.3 selenium and 0.2 cobalt and the diet was fortified with vitamin A (6000 IU), vitamin D (600 IU) and vitamin E (47 IU).

Experimental design and treatments

This experiment was a randomized complete block design with two RUSITEC units, each with eight fermenters resulting in four replicates per treatment (n = 4). The biochar products CP016, CP024 and CP028, were sourced from Cool Planet® (Greenwood Village, CO, USA) and were produced from a common source of jack-pine/yellow pine wood chips. These were manufactured using a patented method (Engineered Biocarbon Technology™) for safe use and were confirmed to be toxin free and approved for inclusion in cattle diets on an experimental basis by the appropriate regulatory agency (Innotech Alberta Inc., Vegreville, AB, CA). The biochar products were pyrolyzed at temperatures ranging from 400-600 °C with residence time intervals of a few minutes. The resulting biochar products differed in bulk density, surface area, pore volume, pH and chemical compositions as reported in Table 1. The control treatment consisted of the basal experimental diet without biochar inclusion [1) Control (TMR only)]. Biochar treatments were included in the basal diet at 20 g kg^{-1} total diet DM [2) TMR + CP016, 3) TMR+ CP024 and 4) TMR + CP028 (Table 2)].

Inoculum source

Four ruminally cannulated Aberdeen Angus-cross heifers were used as rumen fluid donors and were offered the same basal TMR (as fed in the RUSITEC system) ad libitum daily at 0800-0830 h. Rumen contents, both solid and liquid, were collected 2 h post feeding and obtained from four different locations in the rumen. Solid rumen contents (1 kg) were collected, pooled and squeezed to remove excess rumen fluid, composited in a plastic bag and transported immediately to the laboratory. Rumen fluid was placed in pre-warmed thermoses, composited and filtered through three layers of cheese cloth. Excess liquid from the solid contents was filtered through two layers of cheese cloth and mixed with the collected rumen fluid. Prior to dispensing into the RUSITEC fermenters, pH of the fluid was recorded.

Experimental apparatus and incubation

All biochar products and feed ingredients were dried for 48 h at 55 ºC and ground through a 4 mm screen (Arthur Thomas Co., Philadelphia, PA, USA) prior to the experiment. The treatment ingredients were placed in Ankom nylon bags (10 × 20 cm, pore size 50 µm; Ankom technology corp., Macedon, NY, USA) and two RUSITEC (Czerkawski and Breckenridge, 1977) units were used, each equipped with eight anaerobic fermenters (920 mL capacity each). The RUSITEC experiment was conducted for 17 d, with 10 d of adaptation followed by 7 d of sampling. On d 1, each fermenter was placed in a 39 ºC circulating water bath and filled with 200 mL of artificial saliva (McDougall, 1948) and 700 mL of strained rumen fluid. The mixed solid digesta (20 g) and the corresponding treatment bag (10 g DM) were added in separate Ankom bags. Artificial saliva was enriched with $(NH_4)_2SO_4$ (0.3g L^{-1}) and continuously infused (26 mL h^{-1}) via a peristaltic pump to achieve a dilution of 2.9% h^{-1}. The bag with the solid digesta was added only on d 1 and after 24 h, was replaced with a corresponding treatment bag. Thereafter, one treatment bag (incubated 48 h) was replaced daily with a new bag with the corresponding treatment. Anaerobic conditions were achieved by flushing the fermenters with CO_2 during daily bag exchange. The effluent from each fermenter was collected daily in 2 L Erlenmeyer flasks, with gases collected in 2 L reusable gas tight collection bags (Curity®; Conviden Ltd., Mansfield, MA, USA) attached to the effluent flasks.

Measurements

Nutrient disappearance and chemical analysis

Disappearance of DM, OM, CP, NDF, ADF and starch were determined from 48 h incubated bags from d 11 to 15. The analysis of CP disappearance is further described in the protozoa and microbial protein synthesis section. Bags were collected from each fermenter and rinsed under cold water manually until the water ran clear. The bags were then oven dried at 55 ºC for 48 h (AOAC, 1995; method 930.15) and hot-weighed to determine DM disappearance. Residues were composited and pooled over 5 d and ground through a 1 mm screen prior to estimation of OM, NDF and ADF. Approximately 2 g of the ground samples were ball ground using a ball mill (Mixer Mill MM2000; Retsch, Haan, Germany) to measure total N and starch content. Nutrient disappearance was calculated as the difference between the amount of the specific nutrient residue in the diet before and after incubation, divided by the total amount of nutrient in the diet prior to incubation. Ash content was analyzed by combusting samples at 550 °C for 5 h and was used to estimate OM (AOAC 1995; method 942.05). The NDF and ADF contents of the residues were determined using a sequential method (AOAC 1995; method 973.18) with ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA). Heat stable α-amylase (Termamyl®; Novo Nordisk Biochem, Franklinton, NC, USA) and sodium sulfite were used for NDF analysis as described in procedures by Mertens (2002). Starch analysis was completed using an enzymatic approach (Herrerra- Saldana et al. 1990).

Total gas and CH_4 production

Total gas production was measured daily from d 11 to 15 of the sampling period using a gas meter (model DM3A, Alexander-Wright, London, England, UK). From each collection bag, a 20 mL gas sample was obtained with a syringe (20 mL) and injected into exetainers (6.8 mL each; Labco Ltd., Wycombe, England, UK) in duplicate. Methane concentrations were analyzed using gas chromatography (Varian 4900 equipped with GS CarbonPLOT 30 m × 0.32 mm × 3µm column and thermal conductivity detector; Agilent Technologies Canada Inc., Mississauga, ON, CA). The device was equipped with an isothermal oven at a temperature of 35 ºC with helium as the carrier gas (27 cm s^{-1}). Methane production (expressed as % of total gas produced, mg d^{-1}, mg g^{-1} DM disappeared and mg g^{-1} DM incubated) was calculated using total gas production and feed DM (Saleem et al. 2018).

Rumen fermentation characteristics (pH, volatile fatty acids and ammonia nitrogen)

Effluent volume and pH of fermenter fluid of each fermenter were recorded daily using a pH meter (Orion model 260A; Fisher Scientific, Toronto, ON, CA) at the time of bag exchange from d 11 to 15. Subsamples (5 mL) of the effluent collected from the flask, each for volatile fatty acid (VFAs) and ammonia nitrogen (NH_3-N) analyses. For VFA analysis, effluent samples were preserved in 1 mL 25% (wt vol^{-1}) metaphosphoric acid and in 1 mL of H_2SO_4 (1% vol vol^{-1}) for NH_3-N determination. A gas chromatograph (model 5890; Hewlett Packard Lab, Palo Alto, CA, USA) equipped with a 30-m Zebron free FA phase fused silica capillary column (0.32-mm i.d. and 1.0-µm film thickness; Phenomenex, Torrance, CA, USA) was used to determine VFA concentrations (Cottyn and Boucque 1968; Playne 1985). The NH_3-N concentrations were determined as described by Rhine et al. (1998). The concentrations of VFA and NH_3-N (mmol L^{-1}) were multiplied by daily effluent production (L d^{-1}) to determine daily VFA and NH_3-N production (mmol d^{-1}) and the acetic to propionic (A:P) ratio was calculated using the values generated.

Protozoa counts and microbial protein synthesis

Protozoa counts were determined in the collected liquid manually squeezed from bags at the daily treatment-bag exchange on d 11 to 15. Extracted liquid (5 mL) from each bag was mixed with 10% methyl-green-formalin-saline solution (5 mL; 1:1 vol vol^{-1}) as described by Dehority (1993). Samples were stored in the dark at room temperature until counted by light microscopy in a Levy-Hausser counting chamber (Hausser scientific, Horsham, PA, USA).

For microbial protein synthesis, the $(NH_4)_2SO_4$ in McDougall's buffer solution was replaced on d 9 with N^{15}-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO, USA) and infused until the end of the experiment. From d 10 to 15, effluent was collected daily and preserved with 3 mL of sodium azide to achieve a final concentration of 0.1% wt vol^{-1}. Analyses of liquid-associated bacteria (LAB), feed particle-bound bacteria (FPB) and feed particle-associated (FPA) bacteria were conducted via methodology as described by Ribeiro et.al. (2015). On d 16 and 17, a 35 mL sample of the daily effluent was collected from each fermenter and centrifuged (20,000 × g at 4 ºC) to isolate LAB. Thereafter, the pellets collected were washed with phosphate buffer between centrifugations, with this procedure repeated three times. Samples were then suspended in distilled water and lyophilized to determine % N and N^{15}.

The FPA and FPB factions were isolated from 48 h bags from d 16 and 17. Bags were removed from fermenters and gently squeezed to remove excess fluid and were individually placed in separate plastic bags filled with 20 mL of phosphate buffer and processed for 60 s in a Stomacher 400 laboratory blender (Seward Medical Ltd., London, England, UK). The processed liquid was manually squeezed out of the plastic bag into a 50 mL Falcon tube (Fischer Scientific Company, Ottawa, ON, CA) and the bag containing solid feed residues was manually washed two more times with 10 mL of phosphate buffer. The resulting buffer-rinsed fluid from washing the bags was combined with the initially expressed fluid and the total volume was recorded. The expressed fluid containing the FPA bacteria were centrifuged (500 × g at 4º C) for 10 min to remove the washed out feed residues. The supernatant was centrifuged (20,000 × g, at 4 ºC) for 30 min and the new bacterial pellet was washed with phosphate buffer. This process was repeated three times and the pellet was then re-suspended in distilled water. Following processing, all samples (FPA) were frozen at -20 ºC until processing and lyophilized to determine % N and N^{15}.

All feed residues (FPB fraction) were oven dried at 55 ºC for 48 h, weighed for DM determination, ball ground (MM400, RestchInc., Newtown, PA, USA) for N and N^{15} by a combustion analyzer coupled to a mass spectrophotometer (NA1500, Carlo Erba instruments, Milan, Italy) to estimate the microbial N content of FPB. The CP disappearance was calculated by subtracting the microbial mass from feed residues as per the procedures of Ribeiro et al. 2018. Microbial mass was obtained by multiplying microbial N production (mg) in feed residues by the microbial mass per milligram of microbial N (g DM microbial pellet/mg of microbial N). Total microbial N production (mg d^{-1}) was calculated as the sum of microbial nitrogen obtained from LAB, FPA and FPB fractions.

Rumen microbiota: DNA extraction and 16s rRNA copy quantification

The DNA was extracted from FPA, FPB and LPB fractions using a Qiagen QIAmp Stool Mini kit (Qiagen Inc., Toronto, ON, CA) on d 16 to 17. Freeze-dried, mechanically ground bacterial samples (30 mg) were weighed into sterile vials containing zirconia beads (1 mm, 0.3 g; 0.5 mm,0.1 g). Stool lysis buffer (ASL; 1.4 mL) was then added and the samples placed in an ultrasonic homogenizer (Omni Bead ruptor 24; OMNI International, Kennesaw, GA, USA). Samples were heated and mixed in an Eppendorf thermomixer® C (Eppendorf Canada, Mississauga, ON, CA) at 95 °C at 55 × g for 5 min and vortexed prior to centrifuging (20, 000 x g

for 1 min). The supernatant was transferred to a sterile 5 mL microcentrifuge tube (Eppendorf™, Eppendorf Canada, Mississauga, ON, CA) and an inhibitEX® tablet from the Qiagen QIAmp Stool mini kit was added to each tube. Following extraction as per the manufacturer's instructions, the total DNA was quantified using PicoGreen with a NanoDrop 3300 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) to determine DNA quality. A polymerase chain reaction (PCR) was conducted to confirm the presence of the ribosomal gene; thereafter, metagenomic sequencing was conducted to determine bacterial abundance and diversity. Forward (515F;5'-ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA -3') and reverse (806R 5'-TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT-3') primers were used in the sequencing to target the V4 region of 16s rRNA. The PCR reactions were conducted using 1 µL of DNA (1: 10 dilution) in a FastStart High Fidelity PCR System (Roche, Montreal, QC, CA). The initial cycle was conducted at 94 °C (2 min), followed by 33 cycles at three different temperatures (94 °C, 58° C and 72 °C) with 30 s intervals. An elongation step at 72 °C (7 min) was used prior to a second PCR reaction using the FastStart High Fidelity PCR system with the inclusion of Fluidigm barcodes (Fluidigm Corporation, San Francisco, CA, USA). The second PCR reaction was run at a 95 °C (10 min), followed by 15 cycles at 95 °C (15 s), 60 °C (30 s) and 72 °C (1 min). A final elongation step was performed at 72 °C (3 min), with amplification verified by observing products on 2 % agarose gel. Amplified samples were then quantified using Quan-iT™ PicoGreen© dsDNA Assay kit (Life Technologies, Carlsbad, CA, USA), pooled in equal proportions, purified using Ampure XP beads (Beckman Coulter, Mississauga, ON, CA) and quantified into a library using Quan-iT™ PicoGreen© dsDNA Assay kit and Kapa Illumina GA with Revised Primers-SYBR® Fast Universal kit (Kapa Biosystems, Wilmington, MA, USA). The average fragment size was determined using a Labchip GX instrument (PerkinElmer, Waltham, MA, USA) and the library was sent to McGill University and Génome Québec Innovation Centre, Montréal, Canada for sequencing on an Illumina MiSeq (Illumina MiSeq Reagent Kit v2, 500cycle). The sequence and raw fastq files obtained were checked via FastQC program (Andrews 2010) and analyzed using Qiime 2 V2019.1 (Caporaso et al. 2010). The raw files were trimmed (Trimmomatic v0.33) as described by Bolger et al. (2014) to separate good quality reads (phred scores of 33 and above) from short reads (<215,000). The program PEAR v.09.8 (Zhang et al. 2014), was used to merge paired-end reads into a single dataset which was analyzed using Qiime 2 V2019.1 (Caporaso et al. 2010). A DADA2 workflow was used to generate operational

taxonomic units (OTUs); primers, chimeric sequences and OTU clustering were removed using a demultiplexing command via USEARCH61, with an open reference OTU picking approach (Edgar 2010). A denoising algorithm was used to detect possible sequencing errors among samples. A sequence table of the OTUs was constructed and aligned by using PyNAST (Caporaso et al. 2010) and were categorized by using Silva 0.19.1 classifier (Boone et al. 2001; Brenner et al. 2005; Vos et al. 2009; Krieg et al. 2010). A phylogenetic tree was constructed using FastTree (Price et al. 2010). Additionally, the Qiime 2 DADA2 workflow (Bolyen et al. 2019) was used to determine microbial diversity, with Chao1 and Shannon indexes as measures of alpha diversity richness. Beta diversity was determined by Bray-Curtis dissimilarities (Lozupone 2011) and by analyzing phylogenetic distance matrices. The number of OTUs and taxonomic abundance were also evaluated and linear discriminant analysis effect size (LEfSe) was used to determine if specific microbial taxa were affected by treatment. Sequences were deposited to the Small Reads Archive (NCBI) with accession number PRJNA647193.

Statistical analysis

The experiment was analyzed as a randomized complete block design, with treatment and day of sampling as fixed effects and block (RUSITEC units) and fermenters as random effects. Total gas and CH_4 production, VFA and NH_3-N production, protozoa count and bacterial N were analyzed with day of sampling treated as a repeated measure. The covariance structures for these repeated measures were determined based on the minimum Akaike's information criteria value. Data were analyzed using PROC MIXED procedure in SAS (university edition for Linux, SAS Institute, Inc., Cary, NC, USA). Total protozoa counts were log transformed prior to statistical analysis and were tabulated as actual mean counts. Bacterial OTUs and taxonomic abundance were also statistically analyzed using PROC MIXED and false discovery rate corrected *P*-values were generated using a Tukey's test. Residuals were also tested for normality using PROC UNIVARIATE. The type I error rate for testing hypotheses was 0.05 and differences among means were declared significant when *P*-values were < 0.05; moreover, a trend at $0.05 < P < 0.10$ was also assigned.

Results

Nutrient disappearance

Compared to the control, inclusion of biochar in the diet did not affect DM ($P = 0.60$), OM ($P = 0.74$), CP ($P = 0.65$), NDF ($P = 0.42$), ADF ($P = 0.25$) or starch ($P = 0.38$) disappearances (Table 3). There were also no differences in nutrient disappearances among biochar types ($P > 0.05$; Table 3).

Total gas and CH_4 production

Compared to the control, the inclusion of biochar did not affect total gas ($P = 0.97$) or CH_4 production ($P > 0.05$), irrespective of unit of expression. Additionally, CH_4 production did not differ ($P > 0.05$) among biochar treatments.

Rumen fermentation characteristics, protozoa and microbial protein synthesis

Biochar inclusion did not affect rumen media pH compared to the control ($P = 0.77$; Table 5). Moreover, biochar did not affect ($P > 0.05$) the total production of VFA, nor the molar proportions of acetate, propionate and butyrate. Branched VFA production was not affected by biochar ($P = 0.41$) nor was the A:P ($P = 0.51$). Biochar inclusion did not affect NH_3-N production in this study ($P = 0.99$).

Protozoa counts were not affected by biochar inclusion ($P = 0.70$), nor was the amount of total microbial N ($P = 0.60$; Table 6). Additionally, biochar ($P > 0.05$) did not affect LAB, FPB or FPA fractions.

Rumen microbiota

Biochar inclusion did not have an effect on the Chao 1, Shannon index or the number of OTUs ($P > 0.05$; Table 7). A total of 3,699,575 16s rRNA gene sequences were analyzed and classified into 177 families (88.14 % of sequences) and 410 genera (81.70% of sequences). Prevotellaceae, Lachnospiraceae, Veillonellaceae, Spirochaetaceae, Rikenellaceae, Ruminococcaceae, Acidaminococcaceae, Succinovibrionaceae, Fibrobacteraceae and Lactobacillaceae were the ten most predominant families (Table 8). Members of four archaeal families, the Methanomethylophilaceae, Methanobacteriaceae, Methanomicrobiaceae and Methanosarcinaceae were identified. The microbial community structure present in biochar and

control treatments did not differ as denoted by the similar relative abundance of the bacteria families present.

Linear discriminant analysis showed that biochar CP028 increased ($P < 0.05$) the relative abundance of Clostridiaceae 1 whereas, biochar CP024 increased bacterial families F082, UCG-001 and BS11 gut group from the order Bacteroidales (Table 9). Family Pseudomonaceae was increased ($P < 0.05$) in TMR samples supplemented with CP016. A principal coordinate plot of the Bray-Curtis dissimilarities and PERMANOVA pair-wise comparisons on these distance matrices indicated that there were no differences ($P > 0.05$) in the beta diversity among bacterial families.

Discussion

Nutrient disappearance

The inclusion of biochar products differing in physiochemical properties did not affect nutrient disappearance, a finding in agreement with previous *in vitro* (Hansen et al. (2012 and Teoh et al. 2019); and in *vivo* studies (Terry et al. 2019b). Hansen et al. (2012) observed a numerical but non-significant reduction in DMD of a mixed ration-hay diet incubated *in vitro* in buffered rumen fluid with straw-based and wood-based biochars included at 9.0 % of DM. Teoh et al. (2019) also observed a response in DM disappearance *in vitro* when hardwood biochar (3.6 and 7.2 % diet DM) was added to a oaten pasture:maize diet in the RUSITEC. This consistent lack of response may be attributed to the indigestible and inert carbohydrates present in biochar which the rumen microbiota are unable to metabolize (Schmidt and Noack 2000; Teoh et al. 2019). In an *in vivo* study, Terry et al. (2019b) did not observe a response in DM intake or DMD when biochar (CP028) was included at 0.5, 1.0 and 2.0 % diet DM in a barley silage-based TMR offered to Aberdeen Angus heifers. Conversely, Saleem et al. (2018) reported an increase in nutrient disappearance when CP016 was added to a barley silage-based diet in the RUSITEC. The inclusion range of Saleem et al. (2018) was lower (0.5, 1.0, 2.0 % diet DM) than the single level of inclusion (2.0 % diet DM) in the current study. In addition, the level of inclusion in the current study was determined based on the highest level of inclusion implemented by Saleem et al. (2018) so as to obtain the greatest potential responses to biochar from the parameters measured. These responses may indicate that biochar can potentially cause an impediment at higher inclusion levels (9.0 % diet DM) as it is highly indigestible as observed by Hansen et al. 2012. However, this is unlikely as the

levels of biochar in a number of the aforementioned studies in which a response was not observed were relatively low (≤ 2.0 % diet DM). Contrarily, Leng et al. (2012b) also reported an increase in DMD of cassava root and cassava leaf diet *in vitro* in rumen fluid taken from South East Asian cattle adapted for 4 mo to rice hull biochar included at 0.6 % diet DM. The pyrolysis conditions and the source material of the biochar used may have accounted for differences in DMD between their study and ours. In their study, the biomass was pyrolyzed at higher temperatures (600-900 °C) which generated biochar with increased porosity, compared to that used in our study which was pyrolyzed at 400-600 °C. The increased porosity in these biochars was hypothesized to provide microhabitats for microbe populations such as methanotrophs and lignocellulosic degraders (Leng 2014). However, it is difficult to postulate as to how this relatively minor change in porosity would account for such a dramatic difference in microbial activity. In the current study, differences between biochar products in terms of pore volume, surface area and bulk density did not elicit any effect on nutrient disappearance.

Total gas and CH_4 production

Previous biochar studies have reported varying effects on methanogenesis *in vitro* (Hansen et al. 2012; Teoh et al. 2019) and *in vivo* (Terry et al. 2019b; Winders et al. 2019) with some *in vitro* studies reporting a 22-25% decrease in CH_4 production (Leng et al. 2012a, b, c; Saleem et al. 2018). The biochar products also exhibited comparable chemical profiles with each other and had similar carbon and ash content and other parameters that were not reported (electrical conductivity, volatile matter and elemental analyses of H, N and O) with differences primarily in physical properties, which may also have contributed to this response. The differing responses in total gas production and CH_4 emissions in the RUSITEC study of Saleem et al. (2018) and the current study were likely influenced by nutrient disappearance, as the former observed corresponding reduction in CH_4 emissions with their observed increased DM disappearance with the inclusion of biochar CP016, which were not seen in this study. However, in agreement with the current study, Terry et al. (2019b) reported that biochar CP028 did not impact total gas or CH_4 production *in vivo* in heifers fed a barley silage-based TMR diet. In terms of chemical characteristics, the wood-based biochar used by Hansen et al. (2012) in a mixed ration hay diet (*in vitro*) had higher pH (pH 9.6) and carbon (C; 91.2 %) compared to the biochar products used in our study (pH 4.9-7.3; C 73.3-76.9 %), whereas Teoh et al. (2019) used a hardwood biochar (pH 8.2) with a lower carbon content

(10 %). Dietary nutrient disappearance affects fermentation pathways and end products including CO_2 and CH_4 (Winders et al. 2019). Therefore, it is not surprising that total gas and CH_4 results were in agreement with the lack of response in the nutrient disappearance in this study. Pine and wood-based by-products are naturally lignified and can be antinutritive to cattle as these are not readily fermentable to rumen microbes (Frei et al. 2013). Moreover, the pyrolysis step of pine wood chips in biochar production may have combusted the readily digestible sugars and other available nutrients, leaving more recalcitrant carbon and material. These factors render biochar very indigestible, hence the observed lack of response in nutrient disappearance, total gas and CH_4 production.

Biochar has been proposed to reduce enteric CH_4 emissions by mediating redox reactions between methanogens and fermentative consortia (Chen et al. 2014; Kammann et al. 2017) which results in improving biofilm formation, leading to increased DM degradation. Conversely, Terry et al. (2019b) used electron microscopy to demonstrate that biofilms in biochar surfaces are less developed than those in feed substrates. Leng (2014) speculated that biochar in the rumen may play a role in the direct interspecies electron transfer between microbial populations, providing a more efficient and rapid microbial growth between methanotrophs and methanogens. However, the presence of methanotrophs in the rumen are extremely low or may be totally absent (Henderson, et al. 2015; Parmar et al. 2015; Auffret et al. 2018). Further, Terry et al. (2019b) and Teoh et al. (2019), suggested biochar may shift microbial communities but do not significantly affect rumen fermentation, gas and CH_4 production. In the current study, the roles of biochar in facilitating microbial oxidative processes may be negligible due to the low (2.0 % diet DM) inclusion level (Schmidt et al. 2019) and lack of response in DMD disappearance, total gas and CH_4 production and rumen microbiota. On the contrary, Saleem et al. (2018) reported a significant reduction (25.2 %) in CH_4 production (mg g^{-1} DM incubated) in the RUSITEC with a barley silage-based diet with the inclusion of biochar CP016, a product used in our study. However, in their study, the largest reduction was observed when biochar was included at 0.5 % DM. Additionally, Leng et al. (2012a) reported a 24.3 % CH_4 reduction with South East Asian cattle when rice hull biochar was included at 0.6 % DM in a cassava diet. These increasesincreases in DMD, accompanied by a reduction in CH_4 production, suggested that pyrolysis temperature (Leng et al. 2012a, b, c) as well as inclusion level (Saleem et al. 2018) may affect the impact of biochar on the

ruminal microbiota responsible for CH_4 production (Leng 2014), however, the mechanism by which this is accomplished is not clear.

Rumen fermentation characteristics, protozoa and microbial protein synthesis

Rumen media pH was not affected by the differing pH of biochar products and reflected the lack of difference in VFA production among treatments. Molar proportion of VFAs, as well as A:P ratios were not affected by biochar inclusion, also confirmed by McFarlane et al. (2017) who observed no effect of biochar sources (chestnut oak, yellow poplar, and white pine) when included at 8.1 % of diet DM to an orchard grass-based diet *in vitro*. Teoh et al. (2019) observed similar results with hardwood-based biochar added (3.6 and 7.2 % diet DM) to an oaten pasture-mixed hay diet fed to a RUSITEC and suggested that the lack of response was reflective of the unaltered archaeal and bacterial communities. This rationale was apparent in the current study as biochar did not affect microbial community structures as observed by the alpha diversity indices measured and the relative abundance of bacterial families. Terry et al. (2019b) reported similar results *in vivo* with the addition of biochar (0.5, 1.0, 2.0 %) in barley silage-based TMR diet offered to heifers, potentially due to the non-response of DMD to biochar inclusion.

The concentration of NH_3-N was not impacted with biochar inclusion, contrary to findings of Saleem et al. (2018). These authors observed a linear increase (P = 0.06) in NH_3-N concentrations with an increasing inclusion of biochar from 0.5 to 2.0 % of diet DM. Increased ruminal protein degradation as evidenced by increased VFA concentrations and NH_3-N suggested that biochar may have enhanced AA deamination, a response not observed in the current study. Ruminal NH_3-N concentration is a good indicator of the efficiency of conversion of dietary N to microbial N (Firkins et al., 2007) but was not observed in the study as biochar treatments and the control had similar NH_3-N concentrations.

Protozoa populations were unaffected by addition of biochar which agreed with the lack of observed response in fiber (NDF and ADF) disappearance. Protozoa can engulf rumen bacteria, which may increase microbial N cycling (Jouany 1996), resulting in decreased AA supply to the lower intestinal tract (Ivan et al. 1991) and fiber digestion in the rumen (Costa et al. 2010; Abubakr et al. 2013). Similar protozoa numbers for all treatments in the current study are in agreement with the lack of response in NH_3-N, VFA production and rumen microbiota.

Rumen microbiota

Microbial community structures and distribution were unaffected as denoted by the analyses for alpha and beta diversity and consistent with the lack of a change in nutrient disappearance, or gas and CH_4 production when biochar was added to the diet. As biochar did not promote an increase in the bacterial diversity, it is difficult to predict how it would impact the formation of bacterial biofilms within the complete diet. Several bacteria families were observed, of which Prevotellaceae was the most abundant, a result that supports similar observations *in vivo* (Henderson et al. 2015; Terry et al. 2019a, b). Moreover, in the current study, Spirochaetaceae and families from Bacteroidales (BS11 gut group, F082 and UCG-001) were significantly increased in biochar treatments which was also observed by Terry et al. (2019b). Other families observed were Lachnospiraceae and Ruminococcaceae, both of which are normally present in silage and forage-fed animals (Brulc 2009; Henderson et al. 2015). Biochar increased bacteria mostly from Phylum Bacteroidetes (BS11 gut group, F082, UCG-001), Firmicutes (Clostridiaceae 1) and Proteobacteria (Pseudomonaceae). These subpopulations may have been passively trapped or inhabited the biochar due to its porosity. These increases in these bacterial subgroups were in agreement with the *in vivo* study of Terry et al. (2019b) and suggest that biochar may shift subpopulations of the rumen microbiota, but were insufficient in the current study to elicit any significant responses in measured fermentation parameters, including NH_3-N. Rumen microbes utilize NH_3-N (from protein degradation) as a nitrogen source for their growth (Koenig et al. 2000; Bach et al. 2005). The lack of response in ruminal microbiota shifts is further evidenced by the lack of biofilm development in biochar surfaces as observed under electron microscopy (Terry et al. 2019b). The inert, indigestible nature of biochar and its relatively low inclusion in the current study may account for the lack of impact of this additive on fermentation parameters. The archaea observed in the current study comprised less than 2% of the total bacterial families and were similar in samples regardless of biochar type.

Conclusions

The inclusion of biochar differing in physical and chemical characteristics at the studied doses in a barley silage-based TMR had no effect on nutrient disappearance, rumen fermentation, total gas and CH_4 production, microbial protein synthesis or the phylogenetic composition of rumen microbiota. In conclusion, pine-based biochar was not found to be a feed additive that can improve ruminal fermentation and mitigate enteric CH_4 for silage-based TMR diets.

Table 1. Chemical and physical profiles of biochar products.

	Biochar products		
Parameter	CP016	CP024	CP028
Chemical profile			
Carbon, % DM	76.9	73.3	75.0
Fixed carbon, % DM	69.3	71.1	75.0
Ash, %	2.1	1.7	1.0
pH	4.9	7.3	7.0
Physical profile			
Bulk density, kg $(m^3)^{-1}$	287.0	140.0	160.1
Surface area, $m^2\ g^{-1}$	186.0	148.0	250.0
Pore volume, cc g^{-1}	7.36×10^{-2}	6.00×10^{-2}	Not tested

Table 2. Chemical composition (% DM unless stated otherwise) of the control (TMR)[z] and biochar treatments[y].

	Treatments			
Parameter	Control (TMR only)	TMR + CP016	TMR+ CP024	TMR + CP028
DM, %	93.1	93.3	93.5	93.6
OM	90.2	90.2	89.0	90.1
CP	16.5	16.4	16.4	16.5
NDF	33.1	38.3	33.3	37.4
ADF	17.2	17.3	16.9	17.0
Starch	26.5	26.5	26.5	26.5

[z] TMR consisted of 600 barley silage, 270 dry rolled barley grain, 100 canola meal and 30 mineral/vitamin supplement (g kg^{-1} DM basis)

[y] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

Table 3. Nutrient disappearance in control (TMR) and biochar treatments[z] measured over a 5-d sampling period[y].

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[x]
DM	0.66	0.66	0.66	0.64	0.002	0.62	0.60
OM	0.66	0.68	0.65	0.64	0.127	0.33	0.74
CP	0.84	0.85	0.83	0.83	0.092	0.67	0.65
NDF	0.35	0.36	0.31	0.32	0.193	0.12	0.42
ADF	0.27	0.28	0.30	0.28	0.113	0.37	0.25
Starch	0.96	0.96	0.96	0.94	0.073	0.20	0.38

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled from d 11-15. Nutrient disappearance expressed as coefficient of digestibility of dry matter; DMD was calculated from daily treatment bags; OMD, CPD, NDFD, ADFD and starch disappearance were measured from pooled samples

[x] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 4. Total gas and methane production (CH_4) in and control and biochar treatments[z] measured over a 5-d sampling period[y]

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[x]
Total gas production, L d^{-1}	1.8	1.8	1.7	1.8	0.14	0.85	0.97
Methane production							
CH_4, % of total gas production	3.1	3.0	2.9	2.9	0.21	0.49	0.16
CH_4, mg d^{-1}	58.8	66.2	50.6	56.1	10.28	0.66	0.91
CH_4, mg g^{-1} DM incubated	7.0	6.8	6.8	6.7	0.95	0.43	0.48
CH_4, mg g^{-1} DM disappeared	8.1	7.0	8.3	8.4	0.64	0.30	0.27

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled d 11-15

[x] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 5. Rumen media pH, VFA and NH_3-N production in control (TMR) and biochar treatments[z] measured over a 5-d sampling period[y].

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[w]
pH	6.7	6.7	6.7	6.7	0.02	0.51	0.77
VFA production, mmol d^{-1}							
Total VFA,	54.8	52.6	56.2	52.3	4.60	0.49	0.65
Acetate (A),	28.6	27.3	28.3	27.1	3.19	0.54	0.32
Propionate (P),	15.4	14.3	14.9	14.3	1.04	0.75	0.38
Butyrate,	6.8	6.9	6.9	6.9	0.30	0.78	0.78
BCVFA[x],	1.6	1.5	1.6	1.5	0.12	0.70	0.41
A: P ratio	1.8	1.9	1.8	1.9	0.11	0.87	0.51
NH_3-N, mmol d^{-1}	6.8	6.5	6.8	7.0	0.18	0.10	0.99

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled from d 11-15

[x] Branched VFAs, isobutyrate + isovalerate

[w] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 6. Protozoa count and bacterial N in control (TMR) and biochar treatments[z] measured over a 5-d sampling period[y].

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[t]
Protozoa[x], $\times 10^4$/mL	1.4	1.7	1.5	1.5	0.31	0.97	0.70
Bacterial N, mg/d							
Total	63.9	69.7	64.5	64.2	2.46	0.27	0.60
LAB[w]	38.4	41.3	33.7	33.9	2.67	0.13	0.48
FPB[v]	18.8	19.7	21.8	19.7	1.73	0.46	0.32
FPA[u]	7.3	7.8	7.5	9.0	1.16	0.61	0.48

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Protozoa counts sampled d 11-15; Bacterial N sampled d 16-17

[x] *P* values are from log transformed protozoa counts; means presented as back transformed protozoa count/mL

[w] Liquid associated bacteria

[v] Feed particle bound bacteria

[u] Feed particle associated bacteria

[t] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 7. Alpha diversity and richness of bacteria from 16S rRNA gene sequences obtained in control (TMR) and biochar treatments[z] over a 2-d sampling period[y].

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[w]
Chao 1	252.6	231.5	284.6	229.0	26.61	0.74	0.86
Shannon Index	7.3	7.1	7.5	6.9	0.31	0.86	0.47
Number of OTUs[x]	248.9	228.1	277.5	226.7	25.3	0.74	0.83

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled from d 16-17

[x] Operational taxonomical units

[w] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 8. Relative abundance (%) of the top 10 bacteria families present in control (TMR) and biochar treatments[z] over a 2-d sampling period[y].

	Treatments					*P* value	
Parameter	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	SEM	Treatment	Control vs biochar[x]
Prevotellaceae	19.7	24.7	19.2	29.8	7.15	0.43	0.42
Lachnospiraceae	14.9	10.6	18.0	11.4	4.41	0.17	0.59
Veillonellaceae	8.3	8.0	6.5	9.5	2.11	0.36	0.77
Spirochaetaceae	6.0	6.6	6.2	4.5	1.10	0.45	0.88
Rikinellaceae	4.8	5.0	5.9	4.2	0.93	0.40	0.79
Ruminococcaceae	4.6	4.7	5.5	4.1	1.24	0.43	0.56
Acidaminococcaceae	3.5	4.0	3.6	3.7	0.45	0.68	0.34
Succinivibrionaceae	3.4	4.2	3.3	4.6	0.53	0.14	0.16
Fibrobacteraceae	2.4	3.1	2.6	2.0	0.50	0.08	0.44
Lactobacillaceae	2.8	2.7	2.3	3.0	0.73	0.84	0.80

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled on d 16-17

[x] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments

Table 9. Differentially abundant bacteria families in control and biochar treatments[z] evaluated with linear discriminant analysis (effect size) over a 2-d sampling period[y].

Parameter	Relative abundance[x], %				
	Control (TMR only)	TMR + CP016	TMR + CP024	TMR + CP028	LDA score
Clostridiaceae 1	1.62 ± 0.740	2.38 ± 1.900	1.40 ± 1.010	**3.21 ± 1.120**	4.0
Bacteroidales F082	2.64 ± 1.550	1.62 ± 0.636	**2.88 ± 0.789**	1.13 ± 0.875	4.0
Pseudomonadaceae	0.00 ± 0.000	**0.04 ± 0.034**	0.01 ± 0.021	0.01 ± 0.020	3.1
Bacteroidales UCG-001	0.56 ± 0.341	1.04 ± 0.757	**1.68 ± 0.508**	1.05 ± 0.808	3.8
Bacteroidales BS11 gut group	0.19 ± 0.214	0.73 ± 0.323	**0.86 ± 0.514**	0.47 ± 0.331	3.5

[z] Biochar treatments consisted of TMR + biochar (20 g kg^{-1} total diet DM)

[y] Sampled from d 16 and 17

[x] LDA: Linear discriminant analysis. Bold values denote higher abundance of bacteria family in a specific treatment relative to all treatments.

CHAPTER 3

Effects of post-pyrolysis treated biochars on methane production, ruminal fermentation and rumen microbiota of a silage-based diet in an artificial rumen system (RUSITEC)

Abstract

This study investigated the effects of including biochars, which differed in post-pyrolysis treatment, on nutrient disappearance, total gas and CH_4 emissions, rumen fermentation, microbial protein synthesis and rumen microbiota in an artificial rumen system (RUSITEC) fed a barley silage-based diet. The basal diet contained (g/kg dry matter (DM) basis); 600 barley silage, 270 barley grain, 100 canola meal and 30 mineral/vitamin supplement. Three spruced-based biochars were treated post-pyrolysis with either zinc chloride, a hydrochloric acid/nitric acid mixture or sulfuric acid and each included at 20 g/kg of diet DM. Treatments were assigned to sixteen fermenters (n = 4/treatment) in two RUSITEC units in a randomized complete block design. The experiment was conducted over 15 d, with 8 d of adaptation and 7 d of sampling and data collection. Nutrient disappearance of DM, organic matter (OM), acid detergent fiber (ADF), nutrient detergent fiber (NDF) and starch were determined after 48 h of incubation from d 9 to 12 and microbial protein synthesis was measured from d 14 and15. Data were analyzed using PROC MIXED in SAS, with fixed effects of treatment and day of sampling (repeated measure) and random effects of RUSITEC unit and fermenters. Biochar inclusion did not affect disappearance of DM (P = 0.49), OM (P = 0.60), CP (P = 0.47), NDF (P = 0.48), ADF (P = 0.11) or starch (P = 0.58). Moreover, biochar inclusion did not affect total gas production (P = 0.31) or CH_4 produced expressed as mg/g of DM incubated (P = 0.74) and mg/g (P = 0.64), or mg/d (P = 0.70) of DM disappeared. Compared to the control, there was a tendency (P = 0.06) for reduced CH_4 production with biochar inclusion when CH_4 was expressed as percent of total gas produced. Biochar inclusion did not affect total VFA (P = 0.56) or NH_3-N (P = 0.20) production. Microbial protein synthesis was not affected by biochar inclusion (P > 0.05). Total protozoa counts were also unaffected by biochar (P = 0.37) nor did it impact (P > 0.05) the alpha or beta diversity of bacterial populations. In conclusion, the biochars evaluated in this study appeared to have little to no impact on nutrient disappearance, total gas and CH_4 production, rumen fermentation and rumen microbiota in the RUSITEC system.

Introduction

Methane is naturally emitted from wetlands and wildfires as well as a result of anthropogenic activities including manure slurries and enteric fermentation in livestock production (Malone, 2015), with the latter accounting for approximately 5.6% of global anthropogenic GHG emissions (FAO, 2017). Ruminants possess a myriad of microbes consisting of archaea, bacteria, protozoa fungi and bacteriophages that act synergistically within the digestive tract to form biofilms on the surface feed (McAllister et al., 1994; Clokie et al., 2011). Methanogenic archaea are responsible for producing enteric CH_4, which accounts for 2-12% of gross energy intake of the host (Johnson and Johnson, 1995). Dietary manipulation, including the use of feed additives, may be used to reduce emissions (Haque, 2018) by several mechanisms including decreased pH, alterations in digestibility and shifting ruminal fermentation towards increased propionate production which acts as an alternate H_2 sink to the reduction of carbon dioxide to CH_4 by methanogens (Mitsumori and Sun, 2008; Mitsumori et al., 2012).

There is an increasing interest in the use of biochar as a natural growth and feed efficiency enhancer, as a detoxifying agent and as potential CH_4 mitigator (Kammann et al., 2017; Saleem et al., 2018). Biochar is a carbon-rich product arising from the pyrolysis of biomass such as animal wastes, plant residues and lignocellulosic plant materials (Lehmann and Joseph, 2009). It has been primarily used as a soil amendment and has been theorized to have the capability to sequester GHGs, bind toxins and heavy metals and serve as a microhabitat for bacteria such as methanotrophs (Feng et al., 2012; Leng et al., 2014; Joseph et al., 2015a, 2015b). Pyrolysis of biochar forms pores and graphene-like structures of carbon, allowing for microbial populations to form communities that promote inter species electron transfer through biological redox reactions (Kammann et al., 2017). Moreover, chemical activation post-pyrolysis with mineral salts, organic and inorganic acids can increase the pore size and expand the surface area of biochar and add a variety of functional groups (organic acids, phosphate groups) that may impact adsorption and chemical properties (Devi and Saroha, 2016). Biochars that are acidic in nature have been proposed to enhance interspecies H transfer within microbial populations, an outcome that could promote microbial activity and fermentation in the rumen (Leng et al., 2014; Teoh et al., 2019).

It has been hypothesized that the beneficial effects of biochar on microbial communities in soils may also be manifested in the rumen and possibly reduce enteric CH_4 production (Joseph et al., 2015b). However, studies evaluating biochar effects on CH_4 production *in vitro* and *in vivo*

have been inconsistent, ranging from no effect (McFarlane et al., 2017; Terry et al., 2019; Teoh et al., 2019; Winders et al., 2019) to a 24% reduction (Leng et al., 2012a, 2012b, 2012c; Saleem et al., 2018). Hence, this study assessed the impact of biochars treated with salt (zinc chloride) or two acids (hydrochloric acid/nitric acid mixture and sulfuric acid) post-pyrolysis on nutrient disappearance, total gas and CH_4 production, ruminal fermentation and microbiota in an artificial rumen system (RUSITEC) fed a barley silage-based diet.

Materials and Methods

Animal care and handling

The animals used in the study were handled in accordance to the Canadian Council on Animal Care (CCAC, 2009) and the protocols used in animal handling and sampling were reviewed and approved by Agriculture and Agri-Food Canada Lethbridge Research and Development Centre animal care committee.

Experimental design, diet and treatments

A randomized complete block design of four treatments (control plus three biochars) was used with two RUSITEC units (Czerkawski and Breckenridge, 1977), each with eight fermenters providing four replicates per treatment (n = 4). All the biochars were sourced from Stiftelsen for Industriell og Teknisk Forskning (SINTEF) Research Institute, Trondheim, Norway. The biochars were produced by pyrolyzing spruce stem wood chips at 450 °C with a residence time of 3 min and a nitrogen flow rate of 2 L/min to remove pyrolysis vapors. Raw biochar was then divided into 3 subsamples of 2 kg and treated with 1) salt (zinc chloride, $ZnCl_2$); and acids (70% wt%); 2) hydrochloric acid/nitric acid mixture (HCl/HNO_3) or 3) sulfuric acid (H_2SO_4) and left overnight at room temperature. Each subsample of biochar was then desiccated in an oven at 450 °C for 4 h. After desiccation, subsamples were placed in a vacuum filter, moistened with deionized water (3 L per biochar batch), packaged and shipped to the Lethbridge Research and Development Centre. Detailed information on the biochar used was proprietary and was not fully disclosed.

The basal diet was a TMR which consisted of (g/kg DM basis); 600 barley silage, 270 dry rolled barley grain, 100 canola meal and 30 mineral/vitamin supplement. The supplement contained (g/kg DM basis); 565 barley grain, 250 calcium carbonate, 100 canola meal, 30 salt, 25 molasses, 10 premix and 0.66 vitamin E. The premix contained 65 mg zinc, 28 mg manganese, 15

mg copper, 0.7 mg iodine, 0.3 mg selenium and 0.2 mg cobalt (per kg, DM basis). The diet was also fortified with vitamin A (6000 IU); vitamin D (600 IU); and vitamin E (47 IU). The TMR and biochars were oven-dried at 55 °C for 48 h and ground through a 4-mm screen (Wiley Mill, Arthur Thomas Co., Philadelphia, PA, USA). The control diet contained TMR only (10 g DM), with each biochar treatment consisting of TMR (10 g DM) and one of the three biochars included at 20 g/kg of diet. Chemical composition of the control diet and biochar treatments are reported in Table 1.

Inoculum source

Rumen fluid was collected from three ruminally cannulated Aberdeen Angus-cross heifers offered a TMR as per the basal diet. Solid and liquid rumen contents were collected 2 h post-feeding from four different locations within the rumen. Rumen fluid from each cow (4 L per animal) was mixed, filtered through four layers of cheesecloth, pooled and transported immediately to the laboratory where pH was recorded and the fluid kept in a water bath at 39ºC until dispensing into the fermenters. The solid rumen contents (approximately 1 kg) were pooled after sampling, squeezed to remove excess liquid, placed in a plastic bag and immediately transported to the laboratory.

Experimental apparatus and incubations

The RUSITEC experiment was conducted for 15 d, with 8 d for adaptation and 7 d for sampling and data collection. Two RUSITEC units were used, each containing eight 920 mL anaerobic fermenters. On d 1, each fermenter was filled with 200 mL of artificial saliva (McDougall, 1948) and 700 mL of strained rumen fluid; along with two separate polyester bags (10 × 20 cm, pore size 50 µm; Ankom technology corp., Macedon, NY, USA) containing 20 g of solid rumen digesta and 10 g treatment diet. The solid digesta bags were added to the fermenters only on d 1 and were thereafter replaced with corresponding treatment bags. Fermenters were then placed in the RUSITEC water baths which were maintained at 39ºC and artificial saliva (pH 8.2), enriched with $(NH_4)_2SO_4$ (0.3 g/L) continuously infused at a rate of (26 mL/h) via a peristaltic pump to achieve a dilution of 2.9%/h. After 24 h, the bag containing the solid rumen digesta was replaced with corresponding treatment bag containing and thereafter, fermenters were opened daily and one treatment bag (incubated for 48 h) replaced with a new bag with the corresponding treatment. Liquid from the 48-h bags was gently squeezed out prior to replacing with the new

treatment bag and added back to fermenter until needed for sample collection. Anaerobic conditions were achieved by flushing the fermenters with CO_2 during the daily bag exchange. The effluent was collected daily into 2 L Erlenmeyer flasks, whereas gases were collected in 2 L gas tight collection bags (Curity®; Conviden Ltd., Mansfield, MA, USA).

Measurements

Nutrient disappearance and chemical analysis

Disappearance of DM, OM, CP, NDF, ADF and starch was determined using 48 h-incubated treatment bags from d 7 to 11 and were collected on d 9 to 13 of the sampling period. The analysis of CP disappearance is further described in the microbial protein synthesis section. Bags were collected from each fermenter and rinsed under cold water until the water ran clear after which time the bags were then oven dried at 55ºC for 48 h and hot-weighed to estimate DM disappearance (DMD). Residues were composited and pooled over the 5-d sampling period to have sufficient sample for chemical analysis and then ground through a 1-mm screen and analyzed for OM, NDF, ADF and starch content. Nutrient disappearance, expressed as a coefficient of digestibility, was calculated as the difference between the amount of the specific nutrient in the diet substrate before incubation minus that after incubation, divided by the total amount of nutrient in the diet.

Ash content was analyzed by combusting samples at 550 °C for 5 h and used to calculate OM (AOAC, 1995; method 942.05). A sequential method was used to determine NDF (Mertens, 2002) and ADF (AOAC, 1995; method 973.18) using an ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA). Heat stable α-amylase (Termamyl®; Novo Nordisk Biochem, Franklinton, NC, USA) and sodium sulfite were used in the NDF analysis as described by Mertens (2002). Starch analysis was completed using an enzymatic approach as described by Herrerra Saldana et al. (1990).

Total gas and CH_4 production

From each collection bag, gas samples were obtained using a 20 mL syringe and injected into 6.8 mL exetainers (Labco Ltd., Wycombe, England, UK). Gas samples for CH_4 analysis were taken in duplicate to obtain average means for analysis and for checking variability. After taking the gas samples, total gas production was measured daily (d 9 to 13 of sampling period) using a

gas meter (Model DM3A, Alexander-Wright, London, England, UK). A tube was attached to the gas meter and collection bag from which gas was squeezed out; thereafter, the rotations and measurements on the gas meter were obtained, which were used to calculate the amount (L) of gas from the bag. Methane concentrations were analyzed using gas chromatography (Varian 4900 equipped with GS CarbonPLOT 30 m × 0.32 mm × 3µm column and thermal conductivity detector; Agilent Technologies Canada Inc., Mississauga, ON, Canada). The device was equipped with an isothermal oven at 35ºC with helium as the carrier gas (27 cm/s). Methane production (expressed as % of total gas produced, mg/d, mg/g DM disappeared and mg/g DM incubated) was calculated using total gas production and feed DM as described by Saleem et al. (2018).

Rumen fermentation characteristics (pH, VFA and NH_3-N)

Effluent volume and pH in each fermenter were recorded daily during bag exchange from d 9 to 13 of sampling period. Subsamples (5 mL) of effluent were obtained from each fermenter for analyses of volatile fatty acids (VFA) and ammonia nitrogen (NH_3-N). For VFA, effluent samples were preserved in 1 mL 25% (wt/vol) metaphosphoric acid and for NH_3-N, samples were preserved in 1 mL of H_2SO_4 (1% vol/vol) and both stored at -20ºC for subsequent analysis. A gas chromatograph (model 5890A series, Hewlett Packard Lab, Palo Alto, CA, USA) equipped with 30-m Zebron free FA phase fused silica capillary (0.32-mm i.d. and 1.0-µm film thickness column; Phenomenex, Torrance, CA, USA) was used to determine VFA concentrations (Cottyn and Boucque, 1968; Playne, 1985). The NH_3-N concentrations were analyzed as described by Rhine et al. (1998). Concentrations of VFA and NH_3-N (mmol/L) were multiplied by daily effluent production (L/d) to estimate VFA and NH_3-N production (mmol/d) and acetate to propionate (A:P) ratios were also calculated.

Protozoa and microbial protein synthesis

Protozoa were analyzed using liquid squeezed out of the bags incubated for 48 h on d 9 to 13. Approximately 5 mL of extracted liquid from each bag was mixed with 5 mL of 10% methyl-green-formalin-saline solution (Dehority, 1993), with samples stored in the dark at room temperature until counted by light microscopy using a Levy-Hausser counting chamber (Hausser scientific, Horsham, PA, USA).

For microbial N production, $(NH_4)_2SO_4$ in the McDougall's buffer solution was replaced with N^{15}-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO, USA) on d 8 of the sampling period and infused into the fermenters until the end of the experiment. From d 9 to 13, daily effluent was collected and preserved with 3 mL of sodium azide to achieve a final concentration of 0.1% wt/vol. Liquid associated bacteria (LAB), feed particle bound bacteria (FPB) and feed particle associated (FPA) bacteria were collected and analyzed as described by Ribeiro et al. (2015). On d 14 and 15 of sampling period, a 35 mL- sample of the daily effluent was collected from each fermenter and centrifuged at 20,000 × g at 4ºC to isolate LAB. The pellets collected were washed with McDougall's buffer solution (re-suspended and centrifuged at 20,000 × g, for 30 min at 4ºC). These procedures were repeated a total of three times to obtain clean pellets and samples were then suspended in distilled water and lyophilized to determine %N and N^{15}. The FPA and FPB factions were prepared from the treatment bags incubated for 48 h. On d 14 and 15, treatment bags were removed from fermenters and gently squeezed to remove excess fermentation fluid. The treatment bags were then individually placed in separate plastic bags filled with 20 mL of McDougall's buffer solution and processed for 60 s in a Stomacher 400 laboratory blender (Seward Medical Ltd., London, UK). The processed liquid was squeezed from the plastic bag into a 50 mL falcon tube (Fischer Scientific Company, Ottawa, ON, Canada) and the bag containing solid feed residues was manually washed twice with 10 mL of McDougall's buffer solution. The fluid resulting from the buffer rinse was combined with the initially expressed fluid into the falcon tube and the total volume was recorded. The expressed fluid, which contained the FPA bacteria, was centrifuged at 500 × g for 10 min at 4ºC to remove the remaining feed residues (containing FPB). The supernatant from this process was transferred into a centrifuge tube and centrifuged at 20,000 × g for 30 min at 4ºC. The resulting bacterial pellet was re-suspended in McDougall's buffer solution, washed and centrifuged three times, and the final pellet was suspended in distilled water and lyophilized to determine %N and N^{15} content of the FPA fraction. All samples were frozen at -20ºC until analyzed. All feed residues from treatment bags were oven dried at 55ºC for 48 h, weighed for DM determination, ball ground (MM400, RestchInc., Newtown, PA, USA) to determine N and N^{15} content, and was analyzed by combustion analysis coupled with the use of a mass spectrophotometer (NA1500, Carlo Erba instruments, Milan, Italy). Total N was analyzed using a combustion analyzer (NA 2100 Carlo Erba instruments, Milan, Italy) and CP disappearance was obtained by subtracting the microbial mass from feed residue (Ribeiro et al., 2018). The latter

value was was calculated by multiplying microbial N production (mg) in feed residues by the microbial mass per milligram of microbial N (g DM microbial pellet/mg of microbial N). Total microbial N (mg/d) was calculated as the sum of microbial nitrogen from LAB, FPA and FPB bacteria.

Rumen microbiota: DNA extraction and 16s rRNA copy quantification

The DNA was extracted from the bacterial fractions (LAB, FPA and FPB) using a Qiagen QIAmp Stool Mini kit (Qiagen Inc. Canada, Toronto, ON, Canada). Extraction was initiated by weighing 30 mg of the freeze-dried, mechanically ground bacterial samples into sterile vials pre-filled with 1 mm (0.3 g) and 0.5 mm (0.1 g) sterile zirconia beads. Buffer ASL (stool lysis buffer; 1.4 mL) was then added and the mixture was placed in an ultrasonic homogenizer (Omni Bead ruptor 24; OMNI International, Kennesaw, GA, USA). In an Eppendorf thermomixer® C (Eppendorf Canada, Mississauga, ON, Canada), samples were heated and mixed at 95 °C at 55 x g for 5 min, vortexed and then centrifuged at 20, 000 x g for 1 min. The supernatant was transferred to a sterile 5 mL Eppendorf™ microcentrifuge tube (Eppendorf Canada, Mississauga, ON, Canada) and an inhibitEX® tablet from the Qiagen QIAmp stool mini kit was added. Following extraction as per the mini kit protocol, the total DNA was quantified by measuring PicoGreen with a NanoDrop 3300 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A polymerase chain reaction (PCR) was conducted to ensure that the 16sRNA gene could be amplified. Once it was confirmed that samples contained the ribosomal gene, metagenomic sequencing was performed to determine bacterial diversity. Primer 515F (5'-ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT-3') were used to target the V4 region of the 16s rRNA. A 33-cycle PCR was performed on the DNA samples, using 1 µl of a 1:10 dilution and with a FastStart High Fidelity PCR System (Roche, Montreal, QC, Canada). The initial cycle was run for 2 min at 94 °C, followed by 33 cycles at temperatures of 94 °C, 58 °C and 72 °C at 30 s intervals. A final elongation step was then conducted at 72 °C for 7 min to prepare the samples for a second PCR reaction. The second PCR reaction was performed using the FastStart High Fidelity PCR system and was incorporated with Fluidigm barcodes (Fluidigm Corporation, San Francisco, CA, USA). The PCR reaction consisted of an initial cycle run for 10 min at 95 °C, followed by 15 cycles run at 95 °C, 60 °C and 72 °C at cycle times of 15 s, 30 s and

1 min, respectively. A final elongation step was performed at 72 °C for 3 min and samples were then placed in a 2% agarose gel to confirm that the amplification process was successful. Consequently, amplified samples were quantified using Quan-iT™ PicoGreen© dsDNA Assay kit (Life Technologies, Carlsbad, CA, USA) and pooled in equal proportions. These pooled samples were purified using Ampure XP beads (Beckman Coulter, Mississauga, ON, Canada) and then quantified into a library using Quan-iT™ PicoGreen© dsDNA Assay kit and Kapa Illumina GA with Revised Primers-SYBR® Fast Universal kit (Kapa Biosystems, Wilmington, MA, USA). A Labchip GX instrument (PerkinElmer, Waltham, MA, USA) was used to determine average fragment size and the library was sent to McGill University and Génome Québec Innovation Centre, Montréal, Canada for sequencing on Illumina MiSeq using the Illumina MiSeq Reagent Kit v2 (500cycle). The sequence and raw fastq files obtained were checked with the FastQC program (Andrews, 2010) and analyzed using Qiime 2 V2019.1 (Caporaso et al., 2010). The raw files were trimmed by using Trimmomatic v0.33 (Bolger et al., 2014) to separate good quality reads (phred scores of 33 and above) from short reads (< 215,000), which were removed. Paired-end reads were then merged using PEAR v.09.8 (Zhang et al., 2014), combined into a single dataset and analyzed using Qiime 2 V2019.1 (Caporaso et al., 2010), with a DADA2 workflow to generate operational taxonomic units (OTUs). Pre-processing of the samples were done by removing the primers, chimeric sequences and OTU clustering using a demultiplexing command via USEARCH61 with an open reference OTU picking approach (Edgar, 2010). The samples were then run through a denoising algorithm to detect possible sequencing errors and chimeras. A sequence table of the OTUs was constructed and aligned by using PyNAST (Caporaso et al., 2010) and were grouped by using Silva 0.19.1 classifier (Boone et al., 2001; Brenner et al., 2005; Vos et al., 2009; Krieg et al., 2010). A phylogenetic tree was then constructed with FastTree (Price et al., 2010). With the Qiime 2 DADA2 workflow (Bolyen et al., 2019), microbial diversity was also determined with Chao1 and Shannon as measures of alpha diversity richness. Further, Bray-Curtis dissimilarities (Lozupone et al., 2011) were obtained to determine beta diversity and phylogenetic distance matrices. The number of OTUs and taxonomic abundance were also evaluated. Linear discriminant analysis effect size (LEfSe) was used to determine the specific microbial taxa affected by the different treatments in the experiment. Sequences were deposited to the Small Reads Archive (NCBI) with accession number PRJNA635164.

Statistical analysis

Data were analyzed as a randomized complete block design with the PROC MIXED of SAS (SAS Institute, Inc., Cary, NC), with fixed effects of treatment and day of sampling. The RUSITEC apparatus and fermenters were considered random effects. Further, the RUSITEC apparatus units were considered as blocking factors for the fermenters. The covariance structures for repeated measures were determined based on the minimum Akaike's information criteria value. Total gas and CH_4 production, total protozoa count, VFA and NH_3-N production and N from bacterial fractions were analyzed with day of sampling included as a repeated measure. Moreover, as described previously, disappearance of OM, NDF, ADF and starch were from pooled samples, calculated from diet DM and analyzed separately and not as repeated measures. Total protozoa counts were log transformed for statistical analysis and were presented as actual protozoa counts in the tables section. The bacteria OTUs and taxonomic abundance were also analyzed using PROC MIXED and false discovery rate corrected *P* values were determined using Tukey's test. Residuals were also tested for normality using PROC UNIVARIATE. Differences among means were declared significant when $P < 0.05$ and a trend at $0.05 < P < 0.10$ unless otherwise stated.

Results

Nutrient disappearance

Compared to the control diet, the inclusion of biochar did not affect disappearance of DM ($P = 0.49$), OM ($P = 0.60$), CP ($P = 0.47$), NDF ($P = 0.48$), ADF ($P = 0.11$) or starch ($P = 0.58$; Table 2). There were also no differences ($P \geq 0.06$) in any nutrient disappearance parameters among biochar treatments.

Total gas and CH_4 production

Total gas and CH_4 production were not affected ($P \geq 0.31$) by biochar inclusion, however, there was a tendency ($P = 0.06$) for lower CH_4 production, expressed as percentage of total gas production, with biochar compared to the control. Methane production, expressed in mg/d, as well as CH_4 expressed in mg/g of DM incubated and mg/g DM disappeared, was numerically highest with the biochar- HCl/HNO_3 treatment and lowest with biochar-$ZnCl_2$.

Rumen fermentation characteristics, protozoa and microbial protein synthesis

The addition of biochar did not affect media pH compared to the control ($P = 0.50$; Table 4) and did not affect ($P \geq 0.20$) the production of total VFA or NH_3-N. Biochar inclusion did not affect ($P \geq 0.56$) molar proportions of acetate, propionate or butyrate compared to the control. Branched VFA concentrations ($P = 0.66$) and A:P ratio ($P = 0.96$) were also unaffected by biochar inclusion. There were no differences ($P \geq 0.09$) in total protozoa counts, FPB, LAB and total microbial N fractions between control and biochar treatments (Table 5).

Rumen microbiota

Biochar did not affect the alpha diversity indices number of OTUs, Shannon diversity index and Chao1($P \geq 0.74$; Table 6). A total of 3,571,276 16s rRNA gene sequences were analyzed, which were classified into 177 families (88.14 % of sequences) and 410 genera (78.29% of sequences). The top 10 families (in descending order) were Lachnospiraceae, Prevotellaceae, Ruminococcaceae, Spirochaetaceae, Rikenellaceae, Veillonellaceae, Family F082, Acidaminococcaceae, Succinovibrionaceae and Family XIII (Table 7). Four archaeal families were observed, which were Methanomethylophilaceae, Methanobacteriaceae, Methanomicrobiaceae and Methanosarcinaceae. Relative abundance of the Rikinellaceae was higher ($P = 0.01$) with biochar treatments compared to the control.

Compared to the control, biochar-HCl/HNO_3 increased ($P < 0.05$) the relative abundance of CAP-aah99b04, Xanthobacteraceae, Rhizobiaceae and Alicylobacillaceae (Table 8). Bacteroidales BS11 gut group was greater in abundance in biochar-$ZnCl_2$, whereas biochar-H_2SO_4 increased the abundance of Rikinellaceae compared to the other treatments and control. Principal coordinates plot of the Bray-Curtis dissimilarities and PERMANOVA pair-wise comparisons on these distance matrices indicate that there were no difference ($P > 0.05$) in beta diversity among treatments.

Discussion

Nutrient disappearance

In the current study, , addition of biochar to a barley-based TMR did not affect nutrient disappearance parameters. This is in agreement with the study conducted by Winders et al. (2019) who reported a lack of response in nutrient disappearance in growing and finishing steers offered

a corn-silage mixed ration supplemented with pine-based biochar (supplied at 0.8 and 3.0 % diet DM). Similarly, Terry et al. (2019) reported that nutrient disappearance was not affected when pine-based biochar (0.5, 1.0 and 2.0 % diet DM) was included in a barley silage-based diet fed to beef heifers. Consistent lack of response regarding nutrient disappearance may be attributed to the indigestible nature of biochar which is not metabolized by the rumen microbiota (Teoh et al., 2019). However, Hang et al. (2018) used biochar produced from rice husks that were pyrolyzed at 900 °C and when included (0.6% diet DM) to a diet of cassava stems, resulted in increased DM digestibility in goats. This was also observed by Saleem et al. (2018) as greater DMD was observed when biochar was included (highest at 0.5 % diet DM) in barley silage-based TMR in RUSITEC. Increased pore volumes and surface areas are promoted by higher pyrolysis temperatures as large amounts of volatile substances escape, favoring the formation of internal pores within the biochar structure (Paethanom and Yoshikawa, 2012; Rizkiana et al., 2014). Increasing the exposed surface area of digestible plant cell walls can increase microbial growth, leading to increased biofilm formation and greater DM degradation (McAllister et al., 1994). High temperature pyrolysis also causes biochar to assume a more amorphous graphene-like structure, which aids in the electron swapping between VFA oxidizing fermenting bacteria and methanogens microbial groups in metabolic processes (Kalachniuk et al., 1994; Leng, 2014). However, the increase in microbial growth was not supported by this study as bacterial populations and DM disappearance were not affected by biochar inclusion.

Chemical activation is a process of applying specific substances to biochar prior to thermal treatment so as to increase porosity to an extent that is even greater than that achieved by pyrolysis at higher temperatures **(**Sahin et al., 2017). Therefore, these activation steps are proposed to improve porosity and sorption capacity of biochar, which may possibly affect surrounding microbiota as described previously. In this study, a salt ($ZnCl_2$) and two acids (HCl plus HNO_3 and H_2SO4) were used to treat biochar post-pyrolysis. The $ZnCl_2$ has previously been used to chemically activate biochar to produce greater surface area compared to other chemical activation reagents including organic and inorganic acids which can result in enhanced adsorptive properties (Donald et al., 2011; Yayha et al., 2015). The activation mechanism of $ZnCl_2$, as described by Subha and Navasiyam (2009), is associated with lateral bonds in biochar that are broken, creating interspaces in between carbon layers, leading to increased microporosity. The use of acids, such

as HCl/HNO_3 and H_2SO_4 are also implemented in activating biocarbon as they similarly increase surface area and enhance porosity (Yahya, et al., 2015).

Total gas and CH_4 production

In the current study, biochar inclusion did not affect total gas production relative to the control. Gases are by-products of rumen fermentation, produced as result of the degradation and digestion of dietary substrates (Winders et al., 2019), hence the lack of a difference in these gases aligns with lack of a difference in DM disappearance in the current study. This is in agreement with the results of Teoh et al. (2019) who reported comparable gas production values i*n vitro*. However, several factors such as source of rumen fluid, type and preparation of substrate, may influence rumen fermentation and gas production (Oss et al., 2016; Duarte et al., 2017; Ramos et al., 2018), that may have resulted in the differences in these responses. Hansen et al. (2012) reported that *in vitro* total gas production (mL/g DM) was unaffected by addition of biochar (9% diet DM) in a hay-based TMR. Similarly, Pereira et al. (2014) demonstrated gas production was not affected by ensiled biochar (0.5, 1, 2% diet DM) mixed with ryegrass incubated *in vitro* with rumen fluid obtained from grazing cows.

In previous studies, the effect of biochar inclusion on CH_4 production ranged from no effect as seen in the *in vitro* studies by MacFarlane et al. (2017), Teoh et al. (2019) and *in vivo* studies of Terry et al. (2019) and Winders et al. (2019); to a decrease ranging from 22-25% as observed in the RUSITEC by Saleem et al. (2018) and *in vivo* by Leng et al. (2012a, 2012b, 2012c) and Hang et al., (2018). An underlying cause may be differences in experimental models in these studies, including the nature of the experimental design (*in vitro* or *in vivo*) creating variation in fermentation conditions (Teoh et al., 2019). Leng et al. (2012c) reported a 24.3% reduction in CH_4 with 0.6% diet DM inclusion of a rice hull-based biochar whereas Saleem et al. (2018) reported a 25.2% reduction in CH_4 production (mg/g DM incubated) in a RUSITEC system using a silage-based TMR, with greatest response at 0.5% diet DM inclusion. In the current study, CH_4, irrespective of unit of expression, was not affected by biochar inclusion which agreed with the outcomes reported elsewhere (Hansen et al., 2012; Pereira et al., 2014; Terry et al., 2019; Winders et al., 2019). These differences suggest that source and the pyrolysis temperature can influence the overall physiochemical profile of biochar and its consequent effect on ruminal microbiota as described previously (Leng et al., 2012a, 2012b, 2012c; Saleem et al., 2018). The level of inclusion may also have affected the rumen function and microbiota as evident in the increase in nutrient

disappearance in some studies (Leng et al., 2012a, 2012b; Saleem et al., 2018). It could be notable that in the current study, the level of inclusion was determined based on the highest level of inclusion implemented by Saleem et al. (2018) so as to elicit the greatest responses to biochar from the parameters measured. The biochars used in the *in vivo* trial conducted by Leng et al. (2012a, 2012b, 2012c) and Hang et al. (2018) were derived from rice hulls and were pyrolyzed at 900 °C, whereas the biochars used in the current study were pyrolyzed at 450 °C and were included in the diet at a higher level (2% diet DM) compared to the aforementioned literature. Leng et al. (2012c) also observed a 25% higher weight gain in biochar fed South East Asian cattle with reduced CH_4 production (22%), which supports a potential role for biochar in mediating redox reactions between methanogens and fermentative consortia and its effects on DM disappearance as described previously. However, in the current study based on the lack of response in DM disappearance, total gas and CH_4 production, the chemical activation steps post-pyrolysis in the biochar treatments did not appear to have elicited a CH_4 response.

Fermentation characteristics, protozoa and microbial protein synthesis

Compared to the control, biochar inclusion did not affect rumen pH or VFA concentrations. McFarlane et al. (2017) also observed a lack of response of biochar on total and individual VFA concentrations regardless of source (chestnut oak, yellow poplar and white pine) or particle size (< 178 µm and > 178 µm) on fermentation of an orchard grass-based diet *in vitro*. Teoh et al. (2019), who found similar results with biochar included to an oaten pasture-mixed hay diet in the RUSITEC, suggested that the lack of response was associated with no change in the composition of bacterial communities (archaeal, bacterial and fungal), a finding that aligns with our study. Terry et al. (2019) also found that addition of biochar (0.5, 1.0 and 2.0%) to a barley silage-based diet fed to heifers did not affect total or individual VFA concentrations.

The concentration of NH_3-N was also not affected by biochar, however Saleem et al. (2018) observed a linear tendency for NH_3-N concentrations to increase as biochar was increased from 0.5 to 2.0 % of diet DM, which agreed with the increase in DM disappearance and branched VFAs in their study, possibly due to an enhancement in AA deamination. This was contrary to the results of the current experiment, which was in agreement with the non-response in branched VFA and DM disappearance results.

Protozoa numbers were also unaffected by biochar, which is in agreement with Garillo et al. (1994) where no changes in protozoa numbers were observed in goats fed diets with biochar at 0.6% of diet DM. Protozoa prey on rumen bacteria via engulfment causing increased microbial N cycling (Jouany, 1996) and a decrease in the supply of AA to the intestine (Ivan et al., 1991). Therefore, an increase or decrease of protozoa populations may influence the number of rumen bacteria, including lignocellulolytic families which can affect total digestibility of fiber in the rumen (Costa et al., 2010; Abubakr, et al., 2013). The unaltered protozoa numbers in the current study agree with the lack of observed response in NH_3-N and VFA production. Saleem et al. (2018) observed in a RUSITEC study that total microbial N production increased as the level of biochar was increased in a barley silage-based diet. These results suggested that biochar improved microbial protein synthesis as there were also positive responses in nutrient disappearance, VFA and NH_3-N concentrations, however this was not observed in the current study.

Rumen microbiota

Biochar did not affect alpha, beta diversities or richness within the bacterial populations nor the Archaea families in the present study, suggesting that it did not affect the rumen microbial communities. Similarly, Teoh et al. (2019) reported unaltered microbial community structures and diversity in the rumen of Holstein dairy cows fed an oaten-maize mixed diet containing hardwood biochar (3.6 % diet DM). Acidic biochar has been hypothesized to improve the redox potential and increase biofilm development as it may mediate interspecies electron transfer more easily among microbial populations, thereby improving rumen fermentation and energy conversion (Leng et al., 2014; Teoh et al., 2019). This however is unlikely as electron microscopy suggests that biofilms on biochar surfaces are less developed (Terry et al., 2019) than those on more readily digested substrates such as grains (Yang et al., 2018), which agreed with rumen microbiota results.

The relative abundance of dominant bacteria in the rumen are driven by the diet, depending on the presence of cellulose, hemicellulose, starches, sugars, organic acids and protein from the substrate. Bacteroidetes and Firmicutes are part of the core bacterial microbiome (Brulc, 2009), hence the presence of Lachnospiraceae, Prevotellaceae, Ruminoccocaeae, Veillonellaceae, Acidaminoccoccaceae in the current study was expected. This is in agreement with the top families observed by Teoh et al. (2019). Bacteroidales and Ruminoccoccaceae are normally abundant in forage-fed animals (Brulc, 2009), whereas Succinivibrionaceae and Prevotellaceae are more

abundant in the rumen of cattle fed concentrate-based diets. Lactate-utilizing families Lactobacillaceae and Veillonellaceae are main producers of VFA and Succinivibrionaceae and Prevotellaceae are the major producers of propionate in the concentrate-fed rumen (Bryant and Small, 1956; Strobel, 1992; Wang et al., 2012). These bacterial families were not affected by biochar as indicated in the relative abundance and microbial diversities, which is reflective of the lack of response in nutrient disappearance, total gas and CH_4 production, as well as VFA production. This was also evident by the lack of change in NH_3-N concentration as there was no shift in relative abundance of deaminating bacteria families (Prevotellaceae, Succinivibrionaceae, Clostridiaceae). Linear discriminant analysis showed a significant increase in the relative abundance of bacterial families of the Bacteroidetes (BS11 gut group, CAP-aah99b04, Rikinellaceae). One of the bacteria families observed, BS11 gut group, has also been observed to increase when biochar was included in a barley silage-based TMR *in vivo* as reported by Terry et al. (2019). Biochar was dispersed within the liquid fraction of the rumen contents, which may have trapped these bacterial groups passively due to its porous nature (Terry et al., 2019). In addition, the relative abundance (comprising 32.8 % of microbiome) of Bacteroidetes in the rumen as part of the core phyla present (Petri et al., 2013) may have resulted in the presence of these families in the solid and liquid components of the rumen fluid. This may also have been the case for other families (from Firmicutes and Proteobacteria) but the response in these groups did not likely elicit a greater shift in the overall community structure indicated by the diversities and general relative abundances observed.

Conclusions

The addition of these particular biochars that had undergone post-pyrolysis treatment to a barley silage-based TMR had no effect on nutrient disappearance, total gas and CH_4 production rumen fermentation, microbial protein synthesis and rumen microbiota. However, the relative abundance of the Rikinellaceae was increased with biochar as compared to the control. In conclusion, given the lack of observed responses, the use of these particular biochars did not offer CH_4 mitigation potential for TMR-based diets and did not improve ruminal fermentation.

Table 1. Chemical composition (g/kg DM unless otherwise stated) of the control (TMR) and biochar treatments[1].

	Treatments			
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar HCl/HNO_3	Biochar H_2SO_4
DM, g/kg	913	899	901	868
OM	896	893	891	894
CP	165	165	165	165
NDF	381	395	396	400
ADF	169	170	169	170
Starch	285	284	285	286

[1]Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet; DM basis).

Table 2. Nutrient disappearance in control (TMR) and biochar[1] treatments measured over a 5-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
Nutrient disappearance[4]							
DM	0.64	0.64	0.63	0.65	0.012	0.10	0.49
OM	0.67	0.66	0.67	0.67	0.134	0.49	0.60
CP	0.89	0.88	0.88	0.89	0.012	0.83	0.47
NDF	0.36	0.33	0.35	0.35	0.185	0.69	0.48
ADF	0.26	0.24	0.24	0.23	0.108	0.36	0.11
Starch	0.96	0.96	0.96	0.94	0.073	0.12	0.58

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet DM; DM basis).

[2] Sampled d 9 – d 13.

[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.

[4] Nutrient disappearance expressed as coefficient of digestibility of dry matter; DMD was calculated from daily treatment bags; OMD, NDFD, ADFD and starch disappearance were measured from pooled samples.

Table 3. Total gas and methane production (CH_4) in control (TMR) and biochar[1] treatments measured over a 5-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
Total gas production, L/day	1.4	1.4	1.4	1.5	0.08	0.41	0.31
Methane production							
CH_4, % of total gas production	4.0	3.3	3.6	3.5	0.21	0.20	0.06
CH_4, mg/day	59.9	53.8	64.5	67.6	4.54	0.23	0.70
CH_4, mg/g DM incubated	4.7	4.3	4.8	5.2	0.28	0.15	0.74
CH_4, mg/g DM disappeared	8.1	6.8	8.3	8.4	0.64	0.28	0.64

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet; DM basis).

[2] Sampled d 9- d 13.

[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.

Table 4. Rumen pH, VFA and NH_3-N production in control (TMR) and biochar treatments[1] measured over a 5-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
pH	6.7	6.7	6.7	6.7	0.01	0.10	0.50
VFA productions, mmol/d							
Total VFA	56.8	56.5	56.4	60.4	1.54	0.27	0.56
Acetate	29.6	28.2	28.9	31.4	1.14	0.26	0.96
Propionate	16.2	16.1	15.5	17.1	0.55	0.26	0.93
Butyrate	7.2	7.1	7.2	6.9	0.30	0.82	0.77
BCVFA[4]	1.7	1.7	1.6	1.7	0.07	0.87	0.66
Acetate: Propionate ratio	1.8	1.8	1.8	1.8	1.80	0.47	0.96
NH_3-N, mmol/d	6.8	6.5	6.5	6.6	0.19	0.55	0.20

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet DM; DM basis).

[2] Sampled d 9- d 13.

[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.

[4] Branched VFAs, isobutyrate + isovalerate.

Table 5. Protozoa count and microbial N in control (TMR) and biochar treatments[1] measured over a 5-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
Protozoa[4], $\times 10^4$/mL	3.0	3.4	4.1	4.0	0.81	0.67	0.37
Microbial N, mg/d							
Total microbial N	76.8	84.7	84.0	79.2	3.83	0.41	0.20
LAB[5]	56.9	62.3	61.4	56.8	3.01	0.45	0.37
FPB[6]	13.0	15.8	15.8	15.5	1.28	0.38	0.09
FPA[7]	6.9	6.7	6.8	6.9	0.46	0.99	0.88

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet DM; DM basis).

[2] Protozoa counts sampled d 9- d 13; Bacterial N sampled d 14- d 15.

[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.

[4] *P* values are from log transformed protozoa counts for statistical analysis (log protozoa/mL); means presented as actual protozoa count/mL.

[5] Liquid associated bacteria.

[6] Feed particle bound bacteria.

[7] Feed particle associated bacteria.

Table 6. Alpha diversity and richness of bacteria from 16S rRNA gene sequences obtained in control (TMR) and biochar treatments[1] over a 2-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
Chao 1	304.6	293.5	321.5	321.0	21.69	0.65	0.74
Shannon Index	7.7	7.6	7.8	7.7	0.14	0.53	0.86
Number of OTUs[4]	298.2	289.3	313.1	313.4	20.87	0.71	0.74

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet; DM basis).
[2] Sampled on d 14 and 15.
[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.
[4] Operational taxonomical units.

Table 7. Relative abundance (%) of the top 10 bacteria families present in control (TMR) and biochar treatments[1] over a 2-d sampling period[2].

	Treatments				SEM	*P* value	
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3		Treatment	Control vs biochar[3]
Lachnospiraceae	17.4	16.4	17.1	16.9	1.30	0.95	0.69
Prevotellaceae	13.7	11.9	13.4	12.0	2.44	0.86	0.58
Ruminococcaceae	8.3	9.0	9.2	9.5	0.96	0.72	0.32
Spirochaetaceae	6.7	7.3	6.2	7.3	0.97	0.41	0.65
Rikenellaceae	4.8b	6.1a	6.5a	5.7a	0.50	0.05	0.01
Veillonellaceae	5.0	4.5	4.4	3.5	0.89	0.61	0.36
F082 (Order Bacteroidales)	3.8	4.0	4.3	4.2	0.38	0.82	0.43
Acidaminococcaceae	3.1	2.7	3.7	3.4	0.57	0.56	0.73
Succinivibrionaceae	3.2	2.8	3.3	2.2	0.36	0.19	0.31
Family XIII (Order Clostridiales)	2.8	2.5	2.6	2.6	0.42	0.93	0.53

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet DM; DM basis).
[2] Sampled on d 14 and 15.
[3] *P* values obtained from the comparison of TMR mean and the average mean of the three biochar treatments.

Table 8. Differentially abundant bacteria families in control and biochar treatments[1] evaluated with linear discriminant analysis (effect size) over a 2-d sampling period[2].

	Relative abundance[3], %				
Parameter	Control (TMR only)	Biochar $ZnCl_2$	Biochar H_2SO_4	Biochar HCl/HNO_3	LDA score
CAP-aah99b04	0.06 ±0.030	0.04±0.035	0.06±0.030	**0.11±0.065**	3.1
Xanthobacteraceae	0.00 ± 0.000	0.01±0.020	0.00±0.000	**0.04±0.055**	3.4
Rhizobiaceae	0.00± 0.000	0.004±0.009	0.01±0.014	**0.03±0.026**	3.5
Alicyclobacillaceae	0.00± 0.000	0.003±0.009	0.00±0.000	**0.02±0.029**	3.6
Bacteroidales BS11 gut group	0.14± 0.000	**0.50±0.217**	0.37±0.193	0.37±0.166	3.3
Rikenellaceae	4.29± 0.576	5.97±2.932	**6.69±0.858**	6.04±1.034	4.1

[1] Raw biochar subsamples after pyrolysis were post-treated with $ZnCl_2$, HCl/HNO_3 acid mixture and H_2SO_4. Biochar treatments consisted of TMR + biochar (20 g/kg of diet DM; DM basis).
[2] Sampled on d 14 and 15.
[3] LDA: Linear discriminant analysis. Bold values denote higher abundance of bacteria family.

GENERAL DISCUSSION

Nutritional manipulation in livestock production is one of the most successful strategies to reduce CH_4 production and the inclusion of biochar, a carbon rich by-product, in cattle diets has been gaining attention as a potential means to reduce enteric CH_4 (Leng 2014; Duarte et al. 2017; Saleem et al. 2018). Charcoal and/or activated carbon derivatives have been reported in some studies to improve animal health (mostly in chicken and cattle production) as they potentially enhance nutrient intake and remove toxins through adsorption into the blood stream, or retention in surfaces and removed via excretion (O'toole et al. 2016; Toth and Dou 2016). Biochar has been specifically produced as a soil amendment used in agronomic production due to it proposed capability of improving soil fertility. Its application allows the slow release of C into the environment and its porosity aids in alleviating toxins, heavy metals and GHGs constraints in the environment (Feng et al. 2012; Zimmerman and Gao 2013; Kammann et al. 2017). These effects on soils are reported to increase agronomic and agricultural crop yields, particularly in the tropics with limited success in temperate regions (Jeffery et al. 2016). In soils, biochar is reported to assimilate compounds by adsorption due to its surface area and cation exchange capacity (such as phosphates, mineral moieties and organic acids; Bagreev et al. 2001). Moreover, with the affinity to cations biochar in the soil increases the water and nutrient exchange which promotes nutrient cycling and plant growth (Kammann et al. 2015; Schmidt et al. 2015). Biochar has been reported to decrease GHG emissions in paddy soils and is hypothesized to help increase the inherent methanotrophic microbiota (Feng et al. 2012). However, this is not fully conclusive as other soil studies suggest that biochar may not affect CH_4 emissions or may actually increase them (Xie et al. 2013; Yu et al. 2013). The observed differences in CH_4 emissions in these studies may be attributed to the composition of biochar, the source of biomass or pyrolysis conditions (Joseph et al. 2015a, b). Notwithstanding interest in its potential for use in animal production systems, particularly in feed, bedding and liquid manure has increased in recent years (Schmidt et al. 2019). It has been reported that biochar, when combined with silage, can reduce mycotoxins (activated biochar; Galvano et al. 1996), pesticides and butyric acid production concentrations while increasing the quantity of lactic bacteria (pine and corn based biochar; Pereira et al. 2014). Moreover, biochar has been reported to shift microbial populations in cattle digestive systems (Leng et al. 2012a, b, c; Teoh et al. 2019; Terry et al. 2019b), which may be a viable rumen-centric

approach in reducing enteric CH_4 emissions. However, biochar's effects on rumen microbiota vary, as it has been reported to increase populations of a few minor families or have inhibitory effects on specific methanogenic groups in the rumen (Teoh et al. 2019; Terry et al. 2019a, b). This is further supported by results from studies evaluating the effects of biochar on CH_4 production both *in vitro* and *in vivo* which range from no effect (McFarlane et al. 2017; Terry et al. 2019b; Teoh et al. 2019; Winders et al. 2019) to a 25% reduction (Leng et al. 2012a, b, c; Saleem et al. 2018). Little data exists regarding the supplementation of biochar in TMR-based diets for beef cattle hence results from the current study aimed to provide information on the novel supplementation of this feed additive for potential backgrounding diets for Canadian beef production. This thesis evaluated the effects of biochar differing in source, level of inclusion, particle size and post-pyrolysis treatments in a barley silage-based TMR, assessed in four *in vitro* experiments with the hypothesis that the inclusion of biochar to TMR diet will reduce enteric CH_4 production and improve associated parameters.

In the *in vitro* batch culture experiments of Chapter 1, the results show that biochar supplementation in a TMR-based diet, irrespective of source, tended to negatively impact DMD at high (> 13.5 % diet DM) levels of inclusion. This may be partially attributed to the displacement of feed by the biochar, which itself is inert and almost completely indigestible (Teoh et al. 2019). More so, the significant reductions in DMD have been observed in the coconut-based biochar, which likely had a higher lignin content (Welker et al. 2015) than the pine-based biochar. However, at low inclusion levels (2.3 and 4.5 % diet DM), no effect was observed which was in agreement with the results from the subsequent RUSITEC experiments which also had relatively low levels of inclusion (2.0 % diet DM). Further, DMD and disappearance of other nutrients (OM, NDF, ADF, starch) were not affected by biochar in this study. It has been suggested that the porous nature of biochar may provide microhabitats for surrounding microbiota, which may increase the surface area of the substrate exposed to these microorganisms, leading to microbial growth and increased biofilm formation. These responses, if they occur, should increase the amount of DM degraded in the rumen (McAllister et al. 1994). The biochars used in Chapter 1 exhibited similar physiochemical characteristics which can partially explain the similar responses in DMD; however, the underlying effects of pore size, in relation to particle size and distribution is not fully understood as the latter parameters have likely changed upon grinding the biochar. The non-response in DMD was further supported by rumen microbiota data observed in Chapters 2 and 3.

The salt ($ZnCl_2$) and acid (HCl/HNO_3 or H_2SO_4) treated biochars post-pyrolysis, which have potentially higher pore volumes than non-treated biochars (Subha and Navasiyam 2009; Sahin et al. 2017), did not alter the rumen microbial populations in the biofilm. The effects of post-pyrolysis treated biochar in the rumen system and its mechanisms affecting rumen function are not clear/ Additionally, it is difficult to understand how an increase in porosity may alter the DMD of substrate and associated rumen fermentation parameters, especially at relatively low levels of inclusion. The evaluation of biochar particle size did not result in any significant effects in this study, which was supported by the results of McFarlane et al. (2017). These authors did not observe any significant differences in DMD between the biochar and control treatments regardless of source or level of inclusion. However, the DMD was significantly higher in fine particle sized (< 0.178 mm) compared to treatment supplemented with coarse particle sized biochar (> 0.178 mm), suggesting that larger particle size may have impeded rumen kinetics.

In the current study, total gas and CH_4 production were unaffected by biochar supplementation, level of inclusion and particle size and comparisons between the various biochar products did not yield any significant differences. Coconut-based and pine (common source of jack-pine/yellow pine mixture) biochar sources were evaluated in this study utilized in the batch culture and one RUSITEC experiment. Chapters 1 and 2 suggest that chemical (carbon content, pH) and physical (bulk density, pore volumes and particle distribution) characteristics of the biochar products did not have an effect on DMD, which as indicated by the the non-responses in gas and CH_4 production. This has also been observed by Terry et al. (2019), Teoh et al. (2019) and Winders et. al (2019) which further supports that gas and CH_4 enteric pathways are influenced by the disappearance of basal feed substrate. Thus, the indigestible nature of biochar and its relatively low inclusion levels in this study did not offer any potential to affect DMD, likely causing the non-response in these parameters. Biochar differing in post-pyrolysis treatment, with either salt ($ZnCl_2$) or acids (HCl/HNO_3 or H_2SO_4) in Chapter 3 did also not affect total gas, CH_4 or their associated parameters. As described earlier, the post-pyrolysis treatments enhance the porosity and sorption capacity of biochar (Sahin et al., 2017) which are proposed to influence its effects in the rumen, but this was not observed in Chapter 3. Literature suggests that salt treatment post-pyrolysis, particularly $ZnCl_2$, is more commonly used to chemically activate biochar due to the improved microporosity, as lateral bonds within the carbon layers are broken, creating channels that increase porosity (Subha and Navasiyam 2009). This type of activation results in greater biochar surface

area and $ZnCl_2$ treated biochar is reported to be more porous compared to those treated with other chemical activation reagents such as organic (citric acid; Devi and Saroha, 2016) and inorganic (H_2SO_4, KOH, NaOH, K_2CO_3, H_3PO_4, and H_2O_2) activating agents (Subha and Navasiyam 2009; Donald et al. 2011; Yayha et al. 2015). The increased porosity would imply greater potential in shifting microbial populations that can affect rumen fermentation and ultimately total gas, CH_4 and associated parameters. Hence, in theory biochar treated with $ZnCl_2$ would have been more effective in mitigating enteric CH_4 or improving ruminal fermentation as compared to biochars treated with HCl/HNO_3; however, this was not the case in Chapter 3. Moreover, biochar is proposed to reduce CH_4 by enhancing microbial biofilm formation due to its pores being suitable bacterial microhabitats; which would improve the methanotroph to methanogen associations (Leng et al. 2014). This, however, is unlikely in these experiments as biochar was included at low levels, hence suggesting that this mechanism is improbable. This was apparent and further supported as the microbial families observed in Chapters 2 and 3 were unaffected. Biochar also acts as a mediating medium for electron transfer during redox reactions between microbial groups, which can improve microbial growth, leading to biofilm formation that can result in improved DM degradation (Leng et al. 2013). This, in turn can be expected to lower CH_4 due to shorter residence time in rumen; however, this was not supported by the results of this study where no effects on gas or CH_4 were observed, responses in agreement with the studies of Hansen et al. (2012), Teoh et al. (2019) and Terry et al. (2019). These results were further supported by the absence of an effect of biochar on protozoa populations in the RUSITEC experiments. Protozoa form symbiotic associations with methanogens through engulfment or attachment (Jouany and Ushida 1996; Leng et al. 2014), hence the non-response in protozoa was reflective of the lack of effect on CH_4 production and the associated methanogens. The proposed mechanisms of biochar adsorbing enteric GHG or shifting major rumen microbial groups were also not observed, likely due to the inert nature of biochar and the relatively low levels of inclusion in the current studies.

Notwithstanding, some *in vivo* and *in vitro* studies (Leng et al. 2012a, b, c; Saleem et al. 2018) have reported a decrease in CH_4 production. Leng et al. (2012a) suggested that biochar effects on CH_4 reduction are dosage dependent and reported that with the combined use of nitrate it can reduce CH_4 emissions by as much as 49 %. The proposed mechanism of action was as redox-active electron mediator that shifted electrons away from microbial oxidation reactions (ie., oxidation of acetate to CO_2) and donated them to nitrate to be abiotically reduced (Leng et al.

2012a; Saquing et al. 2016). However, it is not clear what mechanism occurs when biochar is supplied alone as the reduction of CH_4 following nitrate supplementation is well established (Leng et al. 2012a; Schmidt et al. 2019). Moreover, Schmidt et al. (2019) also suggested that it is likely improbable that at 0.5 to 1.0 % diet DM biochar inclusion level is enough to efficiently mediate such redox reactions in cattle considering the amount of CH_4 produced per animal, which also concurred with the current study results. The low levels of biochar would not have the capacity to act as a terminal acceptor for all the H_2 produced in the rumen in methanogenesis (Schmidt et al. 2019).

Rumen fermentation parameters were not affected by biochar source, particle size or post-pyrolysis treatment in this study as observed in the pH, VFA and NH_3-N in all of the experiments. Furthermore, in the RUSITEC experiments, microbial protein synthesis was also unaffected. Rumen pH is correlated with the presence of H ions and thus is affected by the molar concentrations of individual VFAs. Hence, the lack of response in total and molar concentrations of VFA reflects the similar pH across biochar and control treatments in this study. This is supported by the *in vitro* results of Pereira et al. (2014) who reported that VFA profiles did not vary between the control (silage only and no biochar) and biochar treatments regardless of original biochar source (corn vs pine) or inclusion level (8.1 vs 18.6 % diet DM). Furthermore, the non-response in VFA profiles was further supported by the unaltered cellulolytic, fibrolytic and fermentative rumen microbial groups (Terry et al. 2019b). Biochar's inert nature renders its resistant to microbial decomposition (Noack and Schmidt 2000) and its recalcitrant carbon rich composition lacks digestible nutrients and volatile substances that microbial groups utilize in the rumen. The concentration of NH_3-N was also unaffected in all of the experiments which was in contrast with the findings of Saleem et al. (2018), who observed a tendency ($P = 0.06$) in NH_3-N concentrations to increase in higher biochar inclusion levels (0.5 to 2.0 % of diet DM). These authors suggested that ruminal protein degradation was enhanced as evidenced by increased VFA concentrations in their study and that the NH_3-N might have increased to due enhanced AA deamination. However, these trends were not observed in the NH_3-N results of the experiments in this study.

The absence of an effect of biochar on rumen microbiota was clearly demonstrated in the RUSITEC experiments of Chapters 2 and 3 and reflects the lack of response in the other measurement parameters. The families present with biochar products differing in source and post-pyrolysis treatment were similar in terms of relative abundance and the 9 out of 10 microbial

famlies were present in both which were: Prevotellaceae, Lachnospiraceae, Veillonellaceae, Spirochaetaceae, Rikenellaceae, Ruminococcaceae, Acidaminococcaceae, Succinovibrionaceae and Fibrobacteraceae. This is likely driven by diet, as both RUSITEC experiments evaluated the same basal TMR silage-based diet. The relative abundance of each family in all treatments was also indifferent, however subpopulations through LEfse analysis indicated a shift in certain subpopulations in both RUSITEC experiments. However, biochar did not result in any major microbial community shifts as evident in the nutrient disappearance, gas and fermentation parameters measured, which agreed with biochar studies that employed microbiota analysis (Teoh et al., 2019; Terry et al., 2019). The inert and indigestible nature of biochar, regardless of post-pyrolysis treatment, still renders it unusable and non-metabolizable by ruminal microorganisms. The proposed mechanisms of biochar in promoting biofilm formation through electron mediating capabilities between microbial redox reactions was not supported by concurrent metagenomic analyses completed in this study as well as existing literature (Teoh et al. 2019; Terry et al. 2019). Moreover, the suggested colonization of microbial populations in biochar pores and surfaces resulting in increased nutrient uptake, feed efficiency and lowered CH_4 production (Leng et al. 2014) is not definitive as evidenced otherwise by electron microscopy, as rumen biofilms are more well-developed in highly digestible feed particles (Yang et al. 2018) as compared to biochar surfaces (Terry et al. 2019b).

The inclusion of biochar as a feed additive in cattle diets is still a relatively novel practice (Nevin et al. 2010; Konsolakis et al. 2015) and its effects on in digestive systems are not conclusive (Klüpfel et al. 2014; Yu et al. 2015). A meta-analysis by Schmidt et al. (2019) reported that biochar does not have any negative or adverse effects on animals, whether it is used as a feed additive or veterinary treatment, or the environment. However, a possible long-term effect may be shifting the digestive microbiome in the animal and the potential adsorption of essential feed compounds (Schmidt et al. 2019). These authors also propose that biochar is potentially selective on what bacteria group it adsorbs (gram-negative or gram-positive) depending on the cell envelope composition and size and not on pathogenicity. Furthermore, pore size of biochar may also influence what strains or microbial groups can inhabit its surfaces (Galvano et al. 1996) and what chemicals can be adsorbed. Therefore, it is not clear as to how biochar can promote growth of "beneficial" bacteria, or rumen bacteria particularly when added to ruminant diets as biochar may also favor the growth of pathogenic bacteria. This is further supported by a report of Naka et al.

(2001) where biochar increased the population of *E. coli* O157:H7 over the native flora in animal digestive systems, suggesting pathogens may generally bound more strongly to biochar. However, this was contrary with most reports on biochar inclusion in animal diets as these show increased the ratio of "beneficial" (*Lactobacilli, Enterococcus, Bifidobacterium sp.*) to pathogenic bacteria populations (Watarai et al. 2008; Choi et al. 2009; Chu et al. 2013). Nothwitstanding, these correlations should be systematically investigated to clearly understand the biochar's capability to be inhabited by microbial populations. Therefore, it might be considered ideal to include biochar inoculated with "beneficial" bacteria (directly or using surfactants) to act as a carrier matrix to be administered to the animal and positively influence the existing microbiome (Naka et al. 2001; Schmidt et al. 2019). Moreover, activation, through post-pyrolysis treatments or steaming, increases the surface area through the creation of micropores (< 2 nm) which may be too small for the bacterial pathogens to inhabit (Galvano et al. 1996; Schmidt et al. 2019). Hence it is ideal to examine the correlations between ideal pore sizes that may bind pathogenic bacteria, while promoting the growth of beneficial microorganisms as this may be an approach to positively enhance rumen biofilms while supressing pathogen growth (Schmidt et al. 2019). Notwithstanding, inclusion of biochar in cattle diets should be based on appropriately formulated rations as implementation of any proposed CH_4 mitigation strategy should be accompanied by an assessment of the whole system GHG output to ensure that reductions in CH_4 are not accompanied by increases in other gases or depressions in animal performance.

Based on the current study, biochar, supplemented on its own did not offer any potential to decrease CH_4 emissions or improve ruminal fermentation.However, there are possible improvements that can be implemented to fully realize biochar's effects, such as when it is included in the diet coupled with other compounds (electron acceptors, CH_4 analogues). Literature suggest that strong positive effects of biochar can be obtained when included with nitrate, decreasing CH_4 up to 49 % (Leng et al. 2012c). This may just be the advent of understanding the proposed mechanisms of biochar in reducing CH_4 and can be further explored with not only nitrate but other electron acceptors (sulfate, fumarate) as well. With this, the lack of knowledge, both *in vitro* and in *vivo*, in terms of biochar and redox reactions will be clearer. Much of the published literature has not shown any significant mitigative effects of biochar; however, consensus is still lacking on the interaction effects of biochar source and optimal level of inclusion to realize its full effects in animal systems. It may also be beneficial to compare biochar and other CH_4 mitigating

feed additives (ionophores) or organic acids and investigate possible individualistic or synergistic effects when administered to verify the speculated mechanisms involving redox reactions and biofilm formation. The mechanisms related to these reductions are speculative; additionally, the proposed electron mediating capacilities of biochar due to its graphene structure is not well understood as evidenced by the metagenomic analyses in the current study as well as in published literature (Teoh et al. 2019; Terry et al. 2019b). The enhancement of biofilm formation, based from the study and published literature, is unlikely as exhibited by the unaffected microbiota and rumen fermentation as evidenced by underdeveloped biofilm colonization on biochar surfaces (Terry et al. 2019b). Metagenomic analyses conducted within biochar studies can be completed in order to confirm the results observed by Leng et al. (2012a, b, c) in south east Asian cattle as there may be variations in rumen microbiome, affecting host-microbe-biochar interactions. These authors used biochars which were silicon-rich due to the original biomass (rice husk), which may have higher electron buffering capacity that can favor the microbial redox reactions occurring in the rumen (Yu et al. 2015). In addition to this, there is also the hypothesis that cattle from south east Asia might have had a higher abundance of denitrifying anaerobic methane oxidizing bacteria, which are a group of methanothrophs (Schmidt et al. 2019), although no sufficient metagenomic analyses and classification have been done in their study. These authors, along with Saleem et al. (2018) have been the only studies to find promising effects of biochar on the rumen to date. These reports, however are essential to filling the gaps in knowledge regarding biochar inclusion in feed as a novel approach in mitigating CH_4 and improving animal productivity as these can serve as the basis of future biochar research with additional methodology (i.e., inclusion/comparison with other feed additives, bacteria and metagenomic analyses, replicating biochar specifications) both *in vitro* and *in vivo*. Research on the effects of post-pyrolysis treated biochars or activated carbon in ruminant systems are also not well-known, hence future work may be conducted to examine the correlation of increased porosity, biofilm formation, and microbial redox reactions. With these future considerations, the potential of biochar and its mechanisms related, and the feasibility of its application to backgrounding beef cattle operations will be more clearly understood.

THESIS CONCLUSIONS

In conclusion, *in vitro* batch culture and RUSITEC experiments in the study demonstrated that biochar, regardless of source, level of inclusion, particle size and post-pyrolysis treatment did not affect gas and CH_4 production and rumen fermentation. Moreover, post-treatment of biochar after pyrolysis also did not affect gas, CH_4 production or rumen fermentation parameters. Biochar also mostly did not affect the phylogenetic composition of the rumen microbiota. The specific chapter conclusions are as follows:

Chapter 1: Effects of biochar source, level of inclusion and particle size on *in vitro* DMD, total gas and CH_4 production and ruminal fermentation parameters in a barley silage-based TMR diet

The inclusion of biochar products differing in source, physical and chemical characteristics, as well as particle size did not affect DMD, total gas and CH_4 production or rumen fermentation in a barley silage-based TMR. Thus, biochar as a feed additive did not demonstrate potential as a strategy to mitigate enteric CH_4 for TMR-based diets.

Chapter 2: Effect of pine-based biochars with differing physiochemical properties on methane production, ruminal fermentation and rumen microbiota in an artificial rumen (RUSITEC) fed barley silage

The inclusion of biochar differing in physical and chemical characteristics at the studied doses in a barley silage-based TMR had no effect on nutrient disappearance, rumen fermentation, total gas and CH_4 production, microbial protein synthesis or the phylogenetic composition of rumen microbiota. In conclusion, biochar was not found to be a feed additive that can improve ruminal fermentation and mitigate enteric CH_4 for silage-based TMR diets.

Chapter 3: Effects of post-pyrolysis treated biochars on methane production, ruminal fermentation and rumen microbiota of a silage-based diet in an artificial rumen system (RUSITEC)

The addition of select biochars that had undergone post-pyrolysis treatment to a barley silage-based TMR had no effect on nutrient disappearance, total gas and CH_4 production rumen fermentation, microbial protein synthesis and rumen microbiota. However, the relative abundance

of the Rikinellaceae was increased with biochar as compared to the control. In conclusion, given the lack of observed responses, the biochar products evaluated in this study did not offer CH_4 mitigation potential for TMR-based diets and did not improve associated parameters.

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