ABSTRACT

TANAKA, K. Inhibition of Ruminal Methanogenesis through Thermodynamically Competitive and Kinetically Direct Inhibitory Strategies. (Under the direction of Dr. Vivek Fellner).

Ruminal methanogenesis plays a key role in the maintenance of ruminal fermentation by serving as a primary hydrogen (H_2) sink. As ruminal microorganisms ferment plant carbohydrates and proteins, gaseous products from organic matter including carbon dioxide (CO_2) and H_2 are liberated. The accumulation of the latter establishes a highly reducing chemical environment in the rumen, in which a group of archaea called methanogens is responsible for the reduction of CO_2 and formate to methane (CH_4). There has been an increasing interest in the inhibition of methanogenesis because of its potential for improving feed efficiency and alleviating environmental burden by the CH_4 emission. Methanogenesis accounts for up to 12% of dietary energy loss in ruminants, and CH_4 is a greenhouse gas (GHG) contributing to global warming. Therefore, successful inhibition of ruminal methanogenesis will benefit the ruminant livestock producers by improving the dietary energy efficiency of livestock and relieving the environmental impact of GHG emissions from the industry.

Given the functional role of methanogenesis as a H_2 sink in the rumen, we hypothesized that the inhibition of methanogenesis must be combined with provision of alternative H_2 sinks, lest the accumulation of H_2 halts microbial fermentation. Therefore, the objective of this study was to investigate the effects of alternative H_2 sinks naturally occurring in plants, nitrate (NO_3^-), sulfate (SO_4^{2-}), and 3-nitro-1-propionate (3NPA), and a synthetic methane analogue, bromochloromethane (BCM), as well as the combinations of these compounds on *in-vitro* methanogenesis and fermentation. It was of additional interest to investigate how energy levels in the basal diets might alter the effect of the alternative H_2 sinks included as dietary additives.

We investigated the anti-methanogenic effects of sodium nitrate ($NaNO_3$) and sodium sulfate ($NaSO_4$) in Experiment 1, 3NPA and BCM in Experiment 2, $NaNO_3$-$NaSO_4$ combination treatment (NS) in Experiment 3, and NS and 3NPA (NSP) and NSP and BCM (NSPB) combination treatments in Experiment 4. All experiments were designed to include 3 basal diets of differing energy levels consisting of the following ratios of forage to concentrate (F:C): 70:30, 50:50, and 30:70. Forage used was alfalfa hay while the concentrate mix contained ground corn and soybean meal. Each combination of feed additives and basal diets were added to 100-mL glass bottles with septum caps. Rumen fluid was obtained from a cannulated Hereford steer (*Bos taurus*) and mixed with artificial saliva on 1:2 ratio. Thirty mL of the rumen inoculum were transferred to each *in-vitro* bottle under a constant stream of CO_2. Bottles were capped immediately after inoculation and allowed to sit for 6, 12, and 24 h (for experiments 1 and 2) and 6 and 24 h (for experiments 3 and 4) in a water bath at 39°C. After 24h of incubation, samples were analyzed for CH_4, pH, NH_3-N, and SCFAs. Each experiment consisted of dietary treatments weighed in duplicate culture bottles and replicated twice (n=2) for statistical analyses.

Inclusion of BCM achieved complete inhibition of methanogenesis both individually and in combination with the other additives. BCM's inhibition of CH_4 was concomitant with decreased acetate and increased propionate and butyrate; valerate increased but only in the presence of the other alternative H_2 sinks. Ammonia-N and pH were increased by individual and combined inclusion of $NaNO_3$. Whereas 3NPA was moderately effective as an anti-methanogen, and $NaSO_4$ alone had no effect on the fermentation pattern, combining the two alternative H_2 sinks had a contrasting additive effect. The observed shift in fermentation pattern of the NSPB treatment may indicate a beneficial metabolic outcome due to its potential increase in microbial protein, gluconeogenic capability, and digestive improvement.

© Copyright 2023 by Kairi Tanaka

All Rights Reserved

Inhibition of Ruminal Methanogenesis through Thermodynamically Competitive and Kinetically Direct Inhibitory Strategies.

by
Kairi Tanaka

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Animal Science

Raleigh, North Carolina
2023

APPROVED BY:

Dr. Vivek Fellner Committee Chair	Dr. Francis de los Reyes
Dr. Robert Rose	

BIOGRAPHY

My name is Kairi Tanaka. I was born and raised in Saitama, Japan. I went to Nihon University Buzan Middle and High Schools in Tokyo. Then, I moved to Southern California to attend California State Polytechnic University, Pomona and majored in Animal Science with a pre-vet/grad school option. My first professional aspiration was to become a veterinarian. However, before deciding between a veterinary or graduate school as part of my career, I worked both as a veterinary assistant at a local veterinary clinic and an undergraduate research assistant in a biology lab. The latter work piqued my interest, so I chose to apply for a graduate school. Since I was interested in studying methanogenesis and its mitigation methods, I contacted Dr. Fellner, who kindly accepted me into his lab.

ACKNOWLEDGMENTS

I thank my advisor, Dr. Vivek Fellner, for his mentoring and support throughout my master's program both academically and intellectually. Thank you for always welcoming me into your office, Dr. Fellner, and hours we spent talking is unforgettable. I have learned so much from you but still have so much more to learn. I also thank Mrs. Sarah Jo McLeod for her dedication and assistance throughout my project. Thank you, Sarah Jo, for supporting me in the lab, and it was great to know that I could always rely on you. I would also like to thank my fellow graduate students as well as staff members in the department of animal science for the sense of community. Starting my graduate program in the middle of COVID-19 pandemic was somewhat daunting, but the welcoming atmosphere in the department alleviated the stress. Last but not least, I thank my graduate committee members, Dr. de los Reyes and Dr. Rose, for joining me in this journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

The basis of ruminal methanogenesis

Methane (CH_4) is a key to tackling two major obstacles in ruminant nutrition: feed efficiency and greenhouse gas (GHG) emission. Methanogenesis is the chemical pathway whereby carbon dioxide (CO_2) and formate as primary substrates are reduced to CH_4 and serves as the primary metabolic pathway to eliminate hydrogen (H_2) from the rumen (Olijhoek et al., 2016; Ungerfeld, 2020). Hydrogen and CO_2 are produced when the structural carbohydrates of plant materials are fermented by cellulolytic microbes in the rumen, and the anaerobic condition of the rumen mainly allows for partial oxidation of carbon to gas and short chain fatty acids (SCFAs) while complete oxidation to CO_2 is limited (Olijhoek et al., 2016; Ungerfeld, 2020).

Given the relevance of the methanogenic substrates to other ruminal fermentation products, bypassing methanogenesis may redirect H_2 to the production of SCFAs as alternative H_2 sinks, thereby supplying metabolizable energy to the ruminant (Van Nevel & Demeyer, 1995; Ungerfeld, 2020). Blaxter and Clapperton (1965) measured a range of the methane production of 6.2-10.8 kcal in both sheep and cattle per 100 kcal of dietary energy intake at maintenance level, which suggests roughly up to 10% loss of energy due to CH_4 production (Mitsumori et al, 2002). Depending on the forage-to-concentrate ratio (F:C), the loss of gross energy from the feed energy intake varies; ruminants on a high concentrate diet lose roughly 2-4% while those on a high forage diet can lose up to 15% (Johnson & Johnson, 1995; Van Nevel & Demeyer, 1996; Anderson et al., 2006). In terms of metabolizable energy, the loss of gross energy is between 2% and 12% in ruminants (Czerkawski 1969; Johnson & Johnson, 1995; McCrabb et al., 1997; Olijhoek et al., 2016). Ruminants utilize dietary energy for growth, milk production, etc., which directly affects the outcome of ruminant livestock production (Westberg et al., 2001). Therefore,

channeling methanogenesis and its loss of energy into alternative metabolic products represents a pivotal chance for improving animal production by way of increased feed efficiency.

The global warming effect of methane and contribution by livestock production

The mitigation of methanogenesis in the rumen drew attention of agricultural and environmental researchers because CH_4 is one of the GHGs contributing to global warming (Hook et al., 2010; Johnson & Johnson, 1995; McCrabb et al., 1997). Several agencies have been established worldwide to investigate the source of GHGs and the altering pattern and trajectory of global climate. Globally, climatologists, meteorologists, and the like congruently attribute the recent rise in the GHG concentration in the atmosphere to human activities such as industrial manufacturing and agricultural production (Oreskes, 2004). The United States Environmental Protection Agency (EPA) releases annual reports of GHG emissions and carbon sinks. According to its 1990-2020 Inventory, the enteric fermentation of livestock was the largest source of anthropogenic CH_4 emissions and accounted for 26.9% of the nation's CH_4 emission in 2020 (EPA, 2022).

Additionally, up to 40% of global anthropogenic CH_4 emissions stems from enteric fermentation and manure management of livestock (Key & Tallard, 2012). Although CH_4 was estimated to be 9.5% of GHG emissions in the US—second to 81.3% of CO_2, the 100-year global warming potential of CH_4 is 25-28 times that of CO_2 (Olijhoek et al., 2016). CH_4 is a potent GHG due to its capability and efficiency of energy absorption from the infrared range of light (Van Nevel & Demeyer, 1995). The potency of CH_4 as a GHG speaks to the urgency of CH_4 mitigation strategies in agriculture sector. The rumen is the primary target for CH_4 mitigation in ruminants since 90% of methane is produced in reticulo-rumen (Murray et al., 1978; Van Nevel & Demeyer, 1995).

Potential methods of methanogenesis mitigation through feed additives

Methanogenesis in the rumen is influenced by a combination of dietary, microbial, and chemical factors such as macro- and micro-nutrient compositions of the feed, quality and processing of the feed, fluctuation in microbial populations and diversity, and so on (Johnson & Johnson, 1995; McCrabb et al., 1997). A multitude of research has since sought potential inhibitory strategies against ruminal methanogenesis by way of nutrient manipulation, feed additives, and chemical inhibitors (Brown et al., 2011; Leng, 2014; Latham et al., 2016). Under the normal rumen condition, methanogenesis is a favored and inevitable pathway as a result of microbial fermentation (Leng, 2014). Therefore, inhibiting methanogenesis requires an intervention of the pathway itself (Chalupa, 1984; Johnson & Johnson, 1995); channeling the spared free energy into more productive pathways would most likely necessitate an alteration of the ruminal environment.

In other words, mitigation of CH_4 must be concomitant with manipulation of ruminal redox potential and microbial populations (Honan et al., 2015). The present work focuses on the use of alternative H_2 sinks found in plants and a synthetic direct inhibitor to explore the effects of thermodynamic and kinetic inhibition of methanogenesis. The compounds of interest in the present study are three alternative H_2 sinks sodium nitrate ($NaNO_3$), sodium sulfate ($NaSO_4$), and 3-nitro-1-propionate (3NPA) and a synthetic methane analogue, bromochloromethane (BCM).

Nitrate as an anti-methanogenic agent: mode of action

Nitro-compounds such as nitrate (NO_3^-) have been shown to mitigate methanogenesis effectively while altering other fermentation parameters (Zhou et al., 2011). There are two modes of action for NO_3^- as an anti-methanogenic agent: first, NO_3^- in the rumen serves as an alternative H_2 sink, thereby redirecting the H_2 flow partially away from methanogenesis to its

reduction (Lee et al., 2015; Olijhoek et al., 2016); second, NO_2^- is toxic to ruminal methanogens, as demonstrated by Iwamoto et al. (2002). The dissimilatory NO_3^- reduction to ammonia (NH_3) involves two steps, where nitrite (NO_2^-) is the intermediate (Jones, 1972). The reduction of NO_3^- to NO_2^- and then to NH_3 is exergonic and thermodynamically more favorable than methanogenesis (Thauer et al., 1977). However, the reduction of NO_2^- to NH_3 is the limiting step; NO_2^- accumulates, thereby exerting its toxicity to methanogens (Iwamoto et al., 2002; Lee et al., 2017; van Lingen et al., 2021).

Nitrate can provide a nutritional function as a non-protein nitrogen (NPN) in the diet via the conversion to ammonia (NH_3), which is the main source of microbial protein synthesis (Owens et al., 1980; Lee et al., 2015; Yang et al., 2016). An in-vitro study by Guo et al. (2009) suggests microbial preference for NO_3^- rather than urea as a NPN source. Ruminal bacteria assimilate NH_3 for amino acid synthesis for growth (Firkins & Reynolds, 2007; Patra, 2015; Jin et al., 2018).

The reduction of nitrate is performed by select ruminal bacteria, of which known to date are *Selenomonas ruminantium* subsp*. lactilytica* TH1, *Veillonella parvula*, *Wolinella succinogenes*, and *Pseudomonas aeruginosa* NWAFUP1 (Stewart et al., 1997; Asanuma et al., 2014; Pang et al., 2021). The enzymes involved in the complete reduction of NO_3^- to NH_3 are the periplasmic nitrate and nitrite reductases, Nap and Nrf, and the cytoplasmic nitrate and nitrite reductases, Nar and Nir (Wang et al., 2019). The periplasmic Nap/Nrf complex has been reported in *W. succinogenes* and *S. ruminantium* subsp. *lactilytica* (Wang et al., 2019) and the cytoplasmic Nar/Nir complex in *S. ruminantium* subsp. *lactilytica* (Yoshii et al., 2003; Kaneko et al., 2015). *P. aeruginosa* NWAFUP1 has been shown to perform anaerobic methane oxidation

coupled with denitrification, whereby the reduction of NO_3^- and NO_2^- is coupled with oxidation of CH_4 through an unknown mechanism (Pang et al., 2021).

Nitrate as an anti-methanogenic agent: *in-vitro* experimental evidence

Inhibition of methanogenesis by NO_3^- inclusion was observed in several *in-vitro* studies (Jones, 1972; Anderson & Rasmussen, 1998; Sar et al., 2005; Bozic et al., 2009; Correa et al., 2017), achieving effective inhibition of methanogenesis up to 94% at 43 mM, 94% at 20 mM, 80% at 10 mM, 63% at 8.6 mM, and 98% at 16 mM NO_3^- inclusion level, respectively. The effect of NO_3^- on fermentation parameters other than CH_4 is inconsistent across the *in-vitro* studies: A:P significantly ($p < 0.05$) increased and butyrate significantly decreased (Anderson & Rasmussen, 1998); NH_3-N increased and propionate decreased significantly (Sar et al., 2005); A:P increased, valerate decreased, and NH_3 increased significantly (Bozic et al., 2009); propionate, butyrate, and NH_3 decreased (Correa et al., 2017). However, the piercing pattern across these observations indicate suppressed amounts of reducing equivalents consumed as calculated by Correa et al. (2017) although, with respect to the consumption of H_2, 1 mol of NH_3 generated from NO_3^- would theoretically equate to 1 mol of CH_4 (Lee et al., 2015).

Sulfate as an anti-methanogenic agent: mode of action

Similar to NO_3^-, sulfate (SO_4^{2-}) can serve as a H_2 sink in the rumen, for which there are two pathways, dissimilatory and assimilatory (Qi et al., 1992; Zhao et al., 2020; Zhao & Zhao, 2022). In the dissimilatory pathway, SO_4^{2-} is converted to sulfide (S^{2-}) via Adenosine-5′-phosphosulfate (APS) and sulfite (SO_3^{2-}) and then to hydrogen sulfide (H_2S; Ungerfeld & Kohn, 2006; Drewnoski et al., 2014; Zhao & Zhao, 2022). The reduction of SO_4^{2-} to H_2S is more thermodynamically favorable ($-\Delta G^o = 234$ kJ/mol; Ungerfeld & Kohn, 2006) than methanogenesis ($-\Delta G^o = 131$ kJ/mol; Thauer et al., 1977), thereby outcompeting for H_2

consumption. In the assimilatory pathway, SO_4^{2-} is reduced to SO_3^{2-} then to S^{2-}, which is then incorporated into S-containing amino acids and cofactors such as pantothenic acid, as well as the utilization of H_2S for the synthesis of thio-compounds such as cysteine, methionine, thiamine, and biotin (Bradley et al., 2011; Zhao et al., 2020; Zhao & Zhao, 2022).

There are a group of sulfate-reducing bacteria (SRB) present in the rumen, albeit in small portion (Callaway et al., 2010), which includes the genera *Desulfotomaculum*, *Desulfovibrio*, *Desulfohalobium*, and *Sulfolobus*. *Desulfobulbus*, and *Desulfuromonas* (Drewnoski et al., 2014; Zhao et al., 2020; Zhao & Zhao, 2022). SRB such as *Desulfovibrio* and *Desulfotomaculum* predominantly utilize the dissimilatory pathway, initializing the reduction of SO_4^{2-} to APS with ATP sulfurylase and subsequently to SO_3^{2-} with APS reductase and finalizing the conversion to H_2S with dissimilatory sulfite reductase complex (Bradley et al., 2011; Singh & Lin, 2015).

Sulfate as an anti-methanogenic agent: *in-vitro* experimental evidence

The effect of SO_4^{2-} inclusion on *in-vitro* ruminal methanogenesis has been investigated by, to name a couple, Patra and Yu (2014) and Wu et al. (2015), achieving 5.3% at 5 mM SO_4^{2-}, 5.6% inhibition of CH_4 at 5.56 g/kg DM (or 1.44 mM) SO_4^{2-} inclusion level, respectively. *In-vitro* SO_4^{2-} inclusion does not exert any significant effects on other fermentation parameters in literature (Patra & Yu, 2014; Wu et al., 2015). Despite the theoretical thermodynamics, reduction of SO_4^{2-} does not offer effective competitiveness for H_2 against methanogenesis (Zhao & Zhao, 2022).

3-nitro-1-propionate as an anti-methanogenic agent: mode of action

In addition to NO_3^-, 3NPA is another nitro-compound capable of inhibiting methanogenesis by serving as a H_2 sink and exerting toxicity to methanogens (Anderson and Rasmussen, 1998; Ochoa-García et al., 2019). The mode of action for 3NPA has not been clearly

elucidated (Ochoa-García et al., 2019) but is assumed to be one of the following1: first proposed by Majak and Cheng (1981) was the cleavage of the nitro group from the compound, releasing propionate and NO_2^- based on the detection of the latter compound in the cultures treated with 3NPA. This was later countered by Anderson et al. (1993), suggesting that the nitro group is instead reduced without cleavage to form *β*-alanine. In the latter study, the accumulation of propionate in 3NPA-treated cultures accounted for less than 10% of 3NPA inclusion while appearance of *β*-alanine was measured in liquid chromatography (Anderson et al., 1993). In the metabolism of 3NPA, a key ruminal bacterium has been identified as *Denitrobacterium detoxificans*, which is an obligate anaerobic bacterium and reduces 3NPA to *β*-alanine and other oxidized nitro-compounds by transferring electrons from hydrogen, lactate, and formate, thus competing with methanogens for reductants (Anderson et al., 1993; Anderson and Rasmussen, 1998; Anderson et al., 2000).

3-nitro-1-propionate as an anti-methanogenic agent: *in-vitro* experimental evidence

The effect of 3NPA on *in-vitro* methanogenesis was first investigated by Anderson and Rasmussen (1998), where 20 mM of 3NPA inhibited 68% of CH_4 compared to the control. Recently, Ochoa-García et al. (2019) showed the dose-dependency of anti-methanogenic effect by 3NPA *in vitro*, wherein 4 levels (3, 6, 9, and 12 mM) of 3NPA inclusion quadratically ($p < 0.01$) reduced CH_4 by 29, 80, 99, and 99%, respectively. Similarly, Anderson et al. (2008) observed a similar reduction in methane production with 12-mM 3NPA supplementation down to the detection limit of 0.10 μmol/mL with and without an addition of formate as an extra reductant. In the study by Anderson and Rasmussen (1998), 3NPA tended to increase acetate, significantly increased propionate and butyrate as well as significant decrease in A:P (Anderson & Rasmussen, 1998), indicating increased reducing potential by the 3NPA inclusion. In more

recent studies by Anderson et al. (2008) and Ochoa-García et al. (2019), similar trends have been observed.

Bromochloromethane as an anti-methanogenic agent: mode of action

Halogenated methane analogues such as BCM have been shown to be most effective in inhibiting methanogenesis (Chalupa, 1984; McCrabb et al., 1997). Wood et al. (1968) studied the reaction of the following six multi-halogenated hydrocarbon compounds on vitamin B_{12}: methylene chloride, chloroform, bromoform, carbon tetrachloride, and Freon 12 (CF_2Cl_2). The reaction involving the above reagents results in halomethylcobalamin derivatives—chloromethylcobalamin, dicholomethylcobalamin, dibromomethylcobalamin, diiodomethylcobalamin, trichloromethylcobalamin, and difluorochloromethylcobalamin, respectively—and thusly offer competitive inhibition of the factor III enzyme of the final methyl-transfer in methanogenesis in *Methanobacillus omelianskii* and the cobamide-dependent methyl-transfer (Wood et al., 1968). Under H_2, photolysis of chloromethylcobalamin derivatives results in the common gaseous product of methyl chloride with a preferential order for the least number of chlorine atoms on the chloromethyl ligand of these analogs (Wood et al., 1968); in other words, chloromethylcobalamin has the highest rate of photolytic cleavage of methyl chloride, dichloromethylcobalamin the second highest rate, and trichloromethylcobalamin the slowest rate. In a couple of studies using methylcobalamin with C-14 isotope as the center carbon atom, the photolysis of the carbon-cobalt bond generated $^{14}CH_3$, which could form methane by reacting with a proton (Wood et al., 1968). Using the same radioactive isotope of carbon -14 in the reagents, Wood et al. (1968) observed no effects of excess methyl chloride on the liberation of methylene chloride from dichloromethylcobalamine, which suggests that methylene dichloride is not the product of carbon-cobamide homolytic cleavage. Wood et al.

(1968) then discovered that the methane enzyme does not use chloromethylcobalamin as a substrate for methyl chloride formation as the enzyme has a specificity for the methyl ligand, which is attested when the methyl transfer is reactivated due to the photolysis of chloroalkyl ligand in the enzyme-inhibitor complex. Therefore, BCM inhibits cobamide-dependent methanogenesis (McCrabb et al., 1997).

Bromochloromethane as an anti-methanogenic agent: *in-vitro* experimental evidence

The anti-methanogenic effect of BCM *in vitro* has been consistently efficacious in multiple studies (Trei & Olson, 1969; Goel et al., 2009), achieving complete inhibition of methanogenesis. The reduction of CH_4 production is concomitant with an increase in H_2 production as well as propionate and butyrate *in vitro* (Trei & Olson, 1969; Goel et al., 2009), indicating increased reducing potential due to the absence of methanogenesis as a H_2 sink.

Combined use of hydrogen sinks in literature

In addition to individual inclusion of the alternative H_2 sinks of interest, several studies have investigated the effect of their combinations on ruminal methanogenesis and fermentation. In a study by Van Zijderveld et al. (2010), the individual and combined inclusions of 26 g/kg of NO_3^- and SO_4^{2-} were examined in sheep, where CH_4 was significantly reduced by up to 15, 32, and 46% by the individual inclusion of NO_3^- and SO_4^{2-} and their combination, respectively, indicating additivity of their anti-methanogenic effects. An *in-vitro* study by Patra and Yu (2014) supports the additivity of anti-methanogenic effects by NO_3^- and SO_4^{2-}, where the combination of 5 mM NO_3^- and SO_4^{2-} significantly reduced CH_4 by 36% whereas the individual NO_3^- and SO_4^{2-} inclusion decreased CH_4 by 30 and 5%, respectively.

Additionally, Correa et al. (2017) investigated the effect of the combination of 8 and 16 mM NO_3^- and 4 and 12 mM 3NPA on *in-vitro* ruminal fermentation, where the combination of

NO_3^- and 3NPA significantly reduced CH_4 by between 95 and 98% regardless of the inclusion level of either additive whether individual or combined. However, this drastic reduction of methanogenesis was concomitant with a significant decrease in total and individual SCFA overall as well as NH_3, indicating inhibition of fermentation (Correa et al., 2017).

Conclusion

In conclusion, the feed additives discussed above can inhibit ruminal methanogenesis and alter fermentation pattern by either offering thermodynamically competitive H_2 sinks or directly inhibiting methanogenesis. Alternative H_2 sinks such as NO_3^-, SO_4^{2-}, and 3NPA all exert varying effects on fermentation pattern that are unique to their modes of action and targeted microbial populations. Among the three H_2 sinks, NO_3^- appear to inhibit methanogenesis most effectively by redirecting H_2 flow from methanogenesis to the reduction of NO_3^- and NO_2^- and by intoxicating the methanogen populations. The combination of NO_3^- and SO_4^{2-} offers an enhanced anti-methanogenic effect on ruminal fermentation compared to their individual use. 3NPA also appears to be an effective methanogenesis inhibitor although its extent is not consistent in literature. On the other hand, BCM is a kinetic inhibitor of the methanogenic pathway and thus inhibits methanogenesis completely. Consistent with the functional role of methanogenesis as a primary H_2 sink, complete inhibition of this pathway by BCM results in accumulation of H_2. The use of these additives and consequential changes in fermentation pattern highlight the economy of H_2 in rumen microbiome, wherein the availability of various redox substrates dictates the budgeting of H_2 and direction of interspecies H_2 transfer.

REFERENCE

Anderson, R. C., Rasmussen, M. A., & Allison, M. J. (1993). Metabolism of the plant toxins nitropropionic acid and nitropropanol by ruminal microorganisms. *Applied and Environmental Microbiology*, *59*(9), 3056–3061. https://doi.org/10.1128/aem.59.9.3056-3061.1993

Anderson, R. C., & Rasmussen, M. A. (1998). Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. *Bioresource Technology*, *64*(2), 89–95. https://doi.org/10.1016/S0960-8524(97)00184-3

Anderson, R. C., Rasmussen, M. A., Jensen, N. S., & Allison, M. J. (2000). Denitrobacterium detoxificans gen. nov., sp. nov., a ruminal bacterium that respires on nitrocompounds. *International journal of systematic and evolutionary microbiology*, *50 Pt 2*, 633–638. https://doi.org/10.1099/00207713-50-2-633

Anderson R. C., Krueger, N. A., Stanton, T. B., Callaway, T. R., Edrington, T. S., Harvey, R. B., Jung, Y. S., & Nisbet, D. J. (2008). Effects of select nitrocompounds on in vitro ruminal fermentation during conditions of limiting or excess added reductant. *Bioresource technology*, *99*(18), 8655–8661. https://doi.org/10.1016/j.biortech.2008.04.064

Asanuma, N., Iwamoto, M., Yoshii, T., & Hino, T. (2004). Molecular characterization and transcriptional regulation of nitrate reductase in a ruminal bacterium, Selenomonas ruminantium. *The Journal of general and applied microbiology*, *50*(2), 55–63. https://doi.org/10.2323/jgam.50.55

Blaxter, K. L., & Clapperton, J. L. (1965). Prediction of the amount of methane produced by ruminants. *British Journal of Nutrition*, *19*(1), 511–522. https://doi.org/10.1079/BJN19650046

Bradley, A. S., Leavitt, W. D., & Johnston, D. T. (2011). Revisiting the dissimilatory sulfate reduction pathway. *Geobiology*, *9*(5), 446–457. https://doi.org/10.1111/j.1472-4669.2011.00292.x

Brown, E. G., Anderson, R. C., Carstens, G. E., Gutierrez-Bañuelos, H., McReynolds, J. L., Slay, L. J., Callaway, T. R., & Nisbet, D. J. (2011). Effects of oral nitroethane administration on enteric methane emissions and ruminal fermentation in cattle. *Animal Feed Science and Technology*, *166–167*, 275–281. https://doi.org/10.1016/j.anifeedsci.2011.04.017

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

Callaway, T. R., Dowd, S. E., Edrington, T. S., Anderson, R. C., Krueger, N., Bauer, N., Kononoff, P. J., & Nisbet, D. J. (2010). Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *Journal of animal science*, *88*(12), 3977–3983. https://doi.org/10.2527/jas.2010-2900

Chalupa, W. (1984). Manipulation of rumen fermentation. *Recent Advances in Animal Nutrition*, 143-160. https://doi.org/10.1016/B978-0-407-01160-1.50013-0

Correa, A. C., Trachsel, J., Allen, H. K., Corral-Luna, A., Gutierrez-Bañuelos, H., Ochoa-Garcia, P. A., Ruiz-Barrera, O., Hume, M. E., Callaway, T. R., Harvey, R. B., Beier, R. C., Anderson, R. C., & Nisbet, D. J. (2017). Effect of sole or combined administration of nitrate and 3-nitro-1-propionic acid on fermentation and Salmonella survivability in

alfalfa-fed rumen cultures in vitro. *Bioresource Technology*, *229*, 69–77. https://doi.org/10.1016/j.biortech.2017.01.012

Czerkawski, J. W. (1969). Methane production in ruminants and its significance. *World Review of Nutrition and Dietetics*, *11*, 240–282.

EPA (2022) Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2020. U.S. Environmental Protection Agency, EPA 430-R-22-003.

Firkins, J. L., Yu, Z., & Morrison, M. (2007). Ruminal Nitrogen Metabolism: Perspectives for Integration of Microbiology and Nutrition for Dairy1, 2. *Journal of Dairy Science*, *90*, E1–E16. https://doi.org/10.3168/jds.2006-518

Goel, G., Makkar, H. P. S., & Becker, K. (2009). Inhibition of methanogens by bromochloromethane: Effects on microbial communities and rumen fermentation using batch and continuous fermentations. *British Journal of Nutrition*, *101*(10), 1484–1492. https://doi.org/10.1017/S0007114508076198

Guo, W. S., Schaefer, D. M., Guo, X. X., Ren, L. P., & Meng, Q. X. (2009). Use of Nitrate-nitrogen as a Sole Dietary Nitrogen Source to Inhibit Ruminal Methanogenesis and to Improve Microbial Nitrogen Synthesis In vitro. *Asian-Australasian Journal of Animal Sciences*, *22*(4), 542–549.

Honan, M., Feng, X., Tricarico, J. M., & Kebreab, E. (2021). Feed additives as a strategic approach to reduce enteric methane production in cattle: Modes of action, effectiveness and safety. *Animal Production Science*. https://doi.org/10.1071/AN20295

Hook, S. E., Wright, A.-D. G., & McBride, B. W. (2010). Methanogens: Methane Producers of the Rumen and Mitigation Strategies. *Archaea*, *2010*, e945785. https://doi.org/10.1155/2010/945785

Iwamoto, M., Asanuma, N., & Hino, T. (2002). Ability of Selenomonas ruminantium, Veillonella parvula, and Wolinella succinogenes to Reduce Nitrate and Nitrite with Special Reference to the Suppression of Ruminal Methanogenesis. *Anaerobe*, *8*(4), 209–215. https://doi.org/10.1006/anae.2002.0428

Jin, W., Meng, Z., Wang, J., Cheng, Y., & Zhu, W. (2017). Effect of Nitrooxy Compounds with Different Molecular Structures on the Rumen Methanogenesis, Metabolic Profile, and Methanogenic Community. *Current Microbiology*, *74*(8), 891–898. http://dx.doi.org.prox.lib.ncsu.edu/10.1007/s00284-017-1261-7

Jin, D., Zhao, S. G., Zheng, N., Bu, D. P., Beckers, Y., & Wang, J. Q. (2018). Urea nitrogen induces changes in rumen microbial and host metabolic profiles in dairy cows. *Livestock Science*, *210*, 104–110. https://doi.org/10.1016/j.livsci.2018.02.011

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

Jones G. A. (1972). Dissimilatory metabolism of nitrate by the rumen microbiota. *Canadian journal of microbiology*, *18*(12), 1783–1787. https://doi.org/10.1139/m72-279

Kaneko, J., Yamada-Narita, S., Abe, N., Onodera, T., Kan, E., Kojima, S., Miyazaki, T., Yamamoto, Y., Oguchi, A., Ankai, A., Ichikawa, N., Nakazawa, H., Fukui, S., Takahashi, M., Yamazaki, S., Fujita, N., & Kamio, Y. (2015). Complete genome sequence of Selenomonas ruminantium subsp. Lactylitica will accelerate further understanding of the nature of the class Negativicutes. *FEMS Microbiology Letters*, *362*(9), fnv050. https://doi.org/10.1093/femsle/fnv050

Key, N., & Tallard, G. (2012). Mitigating Methane Emissions from Livestock: A Global Analysis of Sectoral Policies. *Climatic Change*, *12*, 387-414. https://doi.org/10.1007/s10584-011-0206-6

Latham, E. A., Anderson, R. C., Pinchak, W. E., & Nisbet, D. J. (2016). Insights on Alterations to the Rumen Ecosystem by Nitrate and Nitrocompounds. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/article/10.3389/fmicb.2016.00228

Lee, C., Araujo, R. C., Koenig, K. M., & Beauchemin, K. A. (2015). Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers1,2. *Journal of Animal Science*, *93*(5), 2391–2404. https://doi.org/10.2527/jas.2014-8845

Leng, R. A. (2014). Interactions between microbial consortia in biofilms: A paradigm shift in rumen microbial ecology and enteric methane mitigation. *Animal Production Science*, *54*(5), 519–543. https://doi.org/10.1071/AN13381

Majak, W., & Cheng, K. J. (1981). Identification of rumen bacteria that anaerobically degrade aliphatic nitrotoxins. *Canadian journal of microbiology*, *27*(7), 646–650. https://doi.org/10.1139/m81-099

McCrabb, G. J., Berger, K. T., Magner, T., May, C., & Hunter, R. A. (1997). Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. *Australian Journal of Agricultural Research*, *48*(3), 323–329. https://doi.org/10.1071/a96119

Mitsumori, M., Ajisaka, N., Tajima, K., Kajikawa, H., & Kurihara, M. (2002). Detection of Proteobacteria from the rumen by PCR using methanotroph-specific primers. *Letters in applied microbiology*, *35*(3), 251–255. https://doi.org/10.1046/j.1472-765x.2002.01172.x

Murray, R. M., Bryant, A. M., & Leng, R. A. (1978). Methane production in the rumen and lower gut of sheep given lucerne chaff: effect of level of intake. *The British journal of nutrition*, *39*(2), 337–345. https://doi.org/10.1079/bjn19780043

Ochoa-García, P. A., Arevalos-Sánchez, M. M., Ruiz-Barrera, O., Anderson, R. C., Maynez-Pérez, A. O., Rodríguez-Almeida, F. A., Chávez-Martínez, A., Gutiérrez-Bañuelos, H., & Corral-Luna, A. (2019). In vitro reduction of methane production by 3-nitro-1-propionic acid is dose-dependent1. *Journal of animal science*, *97*(3), 1317–1324. https://doi.org/10.1093/jas/skz012

Olijhoek, D. W., Hellwing, A. L. F., Weisbjerg, M. R., Dijkstra, J., Højberg, O., & Lund, P. (2016). Effect of short-term infusion of hydrogen on enteric gas production and rumen environment in dairy cows. *Animal Production Science*, *56*(3), 466–471. https://doi.org/10.1071/AN15521

Oreskes, N. (2004). The Scientific Consensus on Climate Change. *Science*, *306*(5702), 1686–1686. https://doi.org/10.1126/science.1103618

Owens, D., McGee, M., Boland, T., & O'Kiely, P. (2009). Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat1. *Journal of Animal Science*, *87*(2), 658–668. https://doi.org/10.2527/jas.2007-0178

Pang, J., Liu, L., Liu, X., Wang, Y., Chen, B., Wu, S., Yao, J., & Xu, X. (2021). A novel identified Pseudomonas aeruginosa, which exhibited nitrate- and nitrite-dependent methane oxidation abilities, could alleviate the disadvantages caused by nitrate supplementation in rumen fluid fermentation. *Microbial Biotechnology*, *14*(4), 1397–1408. https://doi.org/10.1111/1751-7915.13726

Patra, A. K. (2015). Urea/Ammonia Metabolism in the Rumen and Toxicity in Ruminants. In A. K. Puniya, R. Singh, & D. N. Kamra (Eds.), *Rumen Microbiology: From Evolution to Revolution* (pp. 329–341). Springer India. https://doi.org/10.1007/978-81-322-2401-3_22

Patra, A. K., & Yu, Z. (2014). Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures in vitro while not adversely affecting feed digestion, fermentation or microbial communities. *Bioresource technology*, *155*, 129–135. https://doi.org/10.1016/j.biortech.2013.12.099

Qi, K., Lu, C. D., Owens, F. N., & Lupton, C. J. (1992). Sulfate supplementation of Angora goats: metabolic and mohair responses. *Journal of animal science*, *70*(9), 2828–2837. https://doi.org/10.2527/1992.7092828x

Takahashi, J., & Young, B. A. (1991). Prophylactic effect of l-cysteine on nitrate-induced alterations in respiratory exchange and metabolic rate in sheep. *Animal Feed Science and Technology*, *35*(1), 105–113. https://doi.org/10.1016/0377-8401(91)90103-Y

Thauer, R. K., Jungermann, K., & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews*, *41*(1), 100–180.

Sar, C., Mwenya, B., Santoso, B., Takaura, K., Morikawa, R., Isogai, N., Asakura, Y., Toride, Y., & Takahashi, J. (2005). Effect of Escherichia coli wild type or its derivative with high nitrite reductase activity on in vitro ruminal methanogenesis and nitrate/nitrite reduction. *Journal of animal science*, *83*(3), 644–652. https://doi.org/10.2527/2005.833644x

Singh, S. B., & Lin, H. C. (2015). Hydrogen Sulfide in Physiology and Diseases of the Digestive Tract. *Microorganisms*, *3*(4), 866–889. https://doi.org/10.3390/microorganisms3040866

Stewart, C. S., Flint, H. J., & Bryant, M. P. (1997). The rumen bacteria. *The Rumen Microbial Ecosystem,* 10–72. doi:10.1007/978-94-009-1453-7_2

Trei, J. E. & Olson, W. A. (1969). Effect of chlorine containing analogues of methane on rumen fermentation. *Journal of Animal Science*, *29*, 173-174.

Ungerfeld, E. M. (2020). Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00589

Ungerfeld, E., & Kohn, R. (2006). The Role of Thermodynamics in the Control of Ruminal Fermentation. In *Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress* (pp. 55–85).

Van Lingen, H. J., Fadel, J. G., Yáñez-Ruiz, D. R., Kindermann, M., & Kebreab, E. (2021). Inhibited Methanogenesis in the Rumen of Cattle: Microbial Metabolism in Response to Supplemental 3-Nitrooxypropanol and Nitrate. *Frontiers in Microbiology*, *12*, 705613. https://doi.org/10.3389/fmicb.2021.705613

Van Nevel, C., & Demeyer, D. (1995). Feed additives and other interventions for decreasing methane emissions. In *Biotechnology in Animal Feeds and Animal Feeding* (pp. 329–349). John Wiley & Sons, Ltd. https://doi.org/10.1002/9783527615353.ch17

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

Wang, X., Tamiev, D., Alagurajan, J., Dispirito, A., Phillips, G., & Hargrove, M. (2019). The role of the NADH-dependent nitrite reductase, Nir, from Escherichia coli in fermentative

ammonification. *Archives of Microbiology*, *201*. https://doi.org/10.1007/s00203-018-1590-3

Westberg, H., Lamb, B., Johnson, K. A., & Huyler, M. (2001). Inventory of methane emissions from U.S. cattle. *Journal of Geophysical Research: Atmospheres*, *106*(D12), 12633–12642. https://doi.org/10.1029/2000JD900808

Wood, J. M., Kennedy, F. Scott., & Wolfe, R. S. (1968). Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry*, *7*(5), 1707–1713. https://doi.org/10.1021/bi00845a013

Wu, H., Meng, Q., & Yu, Z. (2015). Effect of pH buffering capacity and sources of dietary sulfur on rumen fermentation, sulfide production, methane production, sulfate reducing bacteria, and total Archaea in in vitro rumen cultures. *Bioresource technology*, *186*, 25–33. https://doi.org/10.1016/j.biortech.2015.02.110

Yang, C., Rooke, J. A., Cabeza, I., & Wallace, R. J. (2016). Nitrate and Inhibition of Ruminal Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane Emissions from Ruminant Livestock. *Frontiers in Microbiology*, *7*, 132. https://doi.org/10.3389/fmicb.2016.00132

Yoshii, T., Asanuma, N., & Hino, T. (2003). Number of nitrate- and nitrite-reducing Selenomonas ruminantium in the rumen, and possible factors affecting its growth. *Animal Science Journal*, *74*(6), 483–491. https://doi.org/10.1046/j.1344-3941.2003.00142.x

Zhao, Y., & Zhao, G. (2022). Decreasing ruminal methane production through enhancing the sulfate reduction pathway. *Animal Nutrition*, *9*, 320–326. https://doi.org/10.1016/j.aninu.2022.01.006

Zhou, Z., Meng, Q., & Yu, Z. (2011). Effects of Methanogenic Inhibitors on Methane Production and Abundances of Methanogens and Cellulolytic Bacteria in *In Vitro* Ruminal Cultures. *Applied and Environmental Microbiology*, *77*(8), 2634–2639. https://doi.org/10.1128/AEM.02779-10

CHAPTER 1: Effects of sodium nitrate, sodium sulfate, and 3-nitro-1-propionate on ruminal methanogenesis and fermentation

1.1 Abstract

Competition for hydrogen (H_2) alters the rumen environment and decreases methane (CH_4) production. It was thusly hypothesized that addition of a H_2 sink may redirect the flow of H_2 away from methanogenesis in mixed cultures of rumen microorganisms. Three known H_2 sinks used in this study are as follows: sodium nitrate ($NaNO_3$), sodium sulfate ($NaSO_4$), and 3-nitro-1-propionate (3NPA).

The experiment was designed to include three levels of each additive in *in-vitro* batch cultures with three basal diets as substrate as well as a control group excluding the additive and/or dietary treatments. The three levels of $NaNO_3$ (7, 14, & 28 g/kg DM), $NaSO_4$ (3, 6, & 12 g/kg DM), and 3NPA (0.50, 1.0, & 2.0 g/kg DM) were added to 100-mL *in-vitro* fermentation bottles containing respective feed substrate varying in forage-to-concentrate ratio from 70:30, 50:50, to 30:70 (high forage, medium forage, & low forage, respectively). After the inoculation with ruminal fluid from a Hereford steer mixed with artificial saliva, the mixed rumen culture in bottles were incubated at 39ºC for 24 h. Samples were analyzed for CH_4 production and short-chain fatty acids (SCFAs) using gas chromatography, pH using a pH probe, and ammonia-N (NH_3-N) using spectrophotometry.

In comparison to the control, 28 g/kg DM $NaNO_3$ reduced CH_4 production by an average of 90% across all three diets at 24 h of incubation. 3NPA reduced CH_4 by 36% in low forage diet. $NaSO_4$ did not affect CH_4 production. Overall, $NaNO_3$ exhibited potent anti-methanogenic effects as well as alterations of pH, NH_3-N, and SCFAs. The effect of 3NPA on ruminal

fermentation seems to depend on the feed substrate, while $NaSO_4$ did not meaningfully affect fermentation regardless of inclusion levels.

1.2 Introduction

The foundation of ruminant nutrition resides literally and figuratively in the rumen – that is the ruminal microbiome. The rumen houses microbial life, whose economy of H_2 dictates utilization or disposal thereof. In the rumen, electron serves as the energy equivalent of currency; the carrier of electrons is H_2 as reducing equivalent. Therefore, the rumen environment is governed by a simple rule of electron transfer between microorganisms. As the ruminant host ingests plant matter, polymers of plant carbohydrates are hydrolyzed by cellulolytic bacteria to their monomer constituents, which are further fermented by ruminal microbes. In contrast to the simplistic rule of seeking electrons for energy, ruminal microbes establish and communicate through complex networks of metabolites. During the process of microbial fermentation, H_2 is liberated, and electrons move within and across microorganisms to operate the enzymes involved in the transformation of substrates into various products. H_2 as carriers of such electrons will eventually accumulate, which inhibits fermentation due to feedback. This is where methane (CH_4) serves to rid the rumen of H_2 through eructation by the ruminant. Methanogenesis is the biochemical pathway whereby carbon dioxide (CO_2) and formate are reduced to CH_4, which efficiently removes H_2 from the rumen and maintains functional fermentation.

However, eructation of CH_4 is tantamount to loss of energy in ruminant nutrition. Blaxter and Clapperton (1965) measured a range of 6.2-10.8 kcal escaping as CH_4 per 100 kcal of dietary energy in sheep and cattle at maintenance, representing feed inefficiency of approximately 6-10% energy loss. Further studies revealed that cell wall carbohydrate fermentation leads to high CH_4 production compared to soluble carbohydrates (Johnson & Johnson, 1995: Moe and Tyrrell,

1979). In other words, a high-concentrate diet is typically less methanogenic than a high-forage diet.

Environmentally, strategies for reduced enteric CH_4 emissions from the ruminant livestock has been intensively investigated because CH_4 is a potent greenhouse gas, and enteric CH_4 emission from livestock accounts for nearly 26.9% of the total US anthropogenic CH_4 emissions in 2020 (US EPA, 2022). Therefore, mitigation of enteric CH_4 emissions solves nutritional and environmental concerns in ruminant livestock production.

Because of the functional role of CH_4 as a H_2 sink in the rumen, CH_4 production is influenced by the presence or introduction of alternative H_2 sinks such as nitrate, sulfate, oxygen, unsaturated fatty acids, and microbial growth (Johnson & Johnson, 1995). The objective of the present study is to investigate the anti-methanogenic potential of sodium nitrate ($NaNO_3$), sodium sulfate ($NaSO_4$), and 3-nitro-1-propionate (3NPA) in combination with three levels of forage-to-concentrate ratios (F:C) in feed substrate.

1.3 Material and Methods

Feed Additives and Feed Substrates

To test whether individual addition of $NaNO_3$, $NaSO_4$, and 3NPA inhibits *in-vitro* methanogenesis, experiments were designed to include four different levels of each additive and three different diets in *in-vitro* batch cultures of mixed rumen microbes. The four levels on a dry matter basis (DM) of each additive included a control without any additives and are as follows: 0, 7, 14, & 28 g/kg DM for $NaNO_3$; 0, 3, 6, & 12 g/kg DM for $NaSO_4$; and 0, 0.50, 1.0, & 2.0 g/kg DM for 3NPA. The diet was formulated with alfalfa hay and concentrate mix for the following F:C: 70:30 for high forage (HF); 50:50 for medium forage (MF); and 30:70 for low forage (LF). The concentrate mix consisted of a mixture of 48% ground corn, 10% soybean

meal, and vitamin and mineral mix. The ingredients and chemical composition of the basal diets are summarized in tables 2.1 and 2.2, respectively. Each combination of feed additive, additive level, and feed substrate (approx. 1.0 g) were prepared in a 100-mL glass culture bottle in duplicates for sampling at 6, 12, and 24 h of incubation on two separate batch runs, thus a total of 144 bottles (= 4 levels of additive × 3 diets × 3 times × 2 duplicates × 2 runs) per additive (figure 1.1).

Rumen Fluid Collection and Inoculation

Rumen inoculum was prepared by mixing rumen fluid and artificial saliva in a 1:2 ratio. The rumen fluid was collected from a cannulated Hereford steer (*Bos taurus*), whose basal diet was a total mixed ration on a perennial grass pasture at the NCSU Metabolic Unit. The artificial saliva was prepared according to the ruminant salivary composition by McDougall (1948) and consisted of $NaHCO_3$, $NaH_2PO_4 \bullet H_2O$, NaCl, KCl, $CaCl \bullet 2H_2O$, $MgCl_2 \bullet 6H_2O$, and Urea. Thirty mL of rumen inoculum were allocated to each fermentation bottle containing 1.0 g of feed substrate and each level of a feed additive while the bottle was flushed with a stream of CO_2 prior to and during inoculation to maintain anaerobicity. Immediately after the inoculation, the bottles were sealed with rubber-lined septum caps and placed in a water bath at 39ºC for inoculation up to 24 h. After each time period, respective bottles were transferred to ice bath to terminate the *in-vitro* microbial activity.

Analytical Measurements

The present study included the measurements of CH_4, pH, ammonia-N (NH_3-N), and short-chain fatty acids (SCFAs). At the end of 6, 12, and 24 h, CH_4 concentration in the fermentation bottle was measured using a gas chromatography (model CP-3800; Varian, Walnut Creek, CA) using a stainless-steel column packed with Molsieve 5A 45/60 mesh (Supelco Inc.,

Bellefonte, PA). Gas samples were withdrawn from the headspace of each bottle with an air-tight syringe (Hamilton Co., Reno, NV). 70-mL of headspace in the bottle allowed for the collection of gas, and the rubber-lined septum cap for retaining the gas in the headspace while taking samples.

After CH_4 measurement, pH of the culture fluid in the bottle was measured with a pH probe (VWR SympHony – model AR25; Accumet Research, Dual Channel pH/Ion Meter Fisher Scientific). Following pH measurements, culture contents were transferred to a tube and centrifuged at 2,000 rpm for 5 min to separate the solid digesta from the liquid. Four mL of supernatant were aliquoted to a 5.0 mL centrifuge tube and was kept in a freezer at - 20ºC for NH_3-N and SCFA analyses. After thawing the 4.0-mL of rumen fluid, two 1.0-mL aliquots of culture fluids were transferred into separate microcentrifuge tubes for NH_3-N and SCFA analyses.

NH_3-N concentration was calculated using the colorimetric procedure outlined by Beecher and Whitten (1970). Standards containing 0, 4, 8, 12, and 16 μg/mL of NH_3-N were prepared. Samples were centrifuged at 15,000 rpm for 15 minutes to separate any remaining solid particles from the liquid. For the analysis, 5.0 μL of each sample or standards were transferred in duplicates into glass tubes and treated with 100 μL of DI water, and 0.5 mL of phenol and sodium hypochlorite reagents were added. The samples and standards were allowed to react for 30 minutes at room temperature. Following 30 min, 4.0 mL of DI water were added to the sample mixture. The sample was then transferred to a cuvette for absorbance measured at wavelength of 630 nm. The standards were used to determine the concentration of unknown samples.

SCFA concentration was measured by gas chromatography (model CP- 3380; Varian, Walnut Creek, CA) using a fused silica capillary column (Nukol^{TM}; Superlco Inc., Bellefonte, PA). One mL aliquots of culture contents were frozen, thawed, and centrifuged at 15,000 rpm for 15 minutes to separate remaining solid particle from the liquid. The 1.0-mL sample aliquot was treated with 0.2 mL of a metaphosphoric acid, which included 2-ethylbutyrate as internal standard. The sample was then centrifuged at 15,000 rpm for 5 minutes and transferred to a GC vial. The column used in this study detected lactate, acetate, propionate, butyrate, valerate, and isoacids the (isobutyrate and isovalerate).

Statistical Analysis

Data from above measurements was analyzed according to a completely randomized block design using the Mixed Procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Data from 6, 12, and 24 h and HF, MF, and LF diets were analyzed separately. Thus, model included the main effect of treatment. Orthogonal contrasts were used to determine linear or quadratic trends. The replicate variable nested within the batch run was treated as a random effect. Thus, the model is represented by:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(k)} + \varepsilon_{ijk}$$

where

y_{ijk} = each response variable measured,

μ = overall mean,

α_i = fixed effect of level

$\beta_{j(k)}$ = random effect of replicate nested within run

ε_{ijk} = subplot error.

Significant effects were declared at *p-value* ≤ 0.05 and tendencies at ≤ 0.10. The analyzed data was visualized in figures using the following R packages: ggplot2 (Wickham, 2016) and ggpubr (Kassambra, 2020).

1.4 Results

Effects of $NaNO_3$ on In-Vitro CH_4, pH, and NH_3-N by Mixed Rumen Culture

The data for the effect of $NaNO_3$ on CH_4, pH, and NH_3-N are summarized in tables 1.3-1.5 and figures 1.2-1.4, respectively. At 6 h, the level of $NaNO_3$ inclusion showed a significant, quadratic trend in all diets. Particularly, the 28 g/kg DM level of $NaNO_3$ completely inhibited CH_4 in the HF, 88% of the control value in the MF, and 87% in the LF diet (tables 1.3-1.5). At 12 and 24 h of incubation, the level of $NaNO_3$ inclusion showed a linear effect on CH_4 in all diets (tables 1.3-1.5; figure 1.2) except for the quadratic effect in the MF diet at 24 h (table 1.4). Across all the diets and hours, the most effective reduction of CH_4 was observed with the 28 g /kg DM inclusion level of $NaNO_3$ compared to the control. At 24 h of incubation, the 28 g /kg DM inclusion level of $NaNO_3$ achieved 96, 88, and 90% reduction of CH_4 compared to the control.

Similar to the effect on CH_4, the level of $NaNO_3$ inclusion had a linear effect on pH with the HF and LF diets (tables 1.1 & 1.3; figure 1.3) and a quadratic effect with the MF diet (table 1.2; figure 1.3). pH in the culture fluid was increased by the $NaNO_3$ inclusion across all the levels compared to the control, and the elevation of pH compared to the control was maintained throughout the incubation period.

The level effect of $NaNO_3$ inclusion on NH_3-N exhibited a linear trend across the diets after 12 h of incubation with the HF and LF diets (tables 1.1 & 1.3; figure 1.3) and only after 24 h with the MF diet (table 1.2; figure 1.3). However, significant effects of $NaNO_3$ inclusion on

NH_3-N were observed at 12 h with the HF and LF diets and 24 h with the MF diet. The combination of $NaNO_3$ and the HF diet tended to increase NH_3-N quadratically at 6 h and linearly at 24 h.

Effects of $NaNO_3$ on SCFAs as In-Vitro Fermentation Products

The data for the effect of $NaNO_3$ on SCFA are summarized in tables 1.6-8. The level effect of $NaNO_3$ inclusion in the HF diet on total SCFA showed a linear trend at 12 h of incubation and a quadratic trend at 24 h (table 1.6). At 12 h of incubation with the HF diet, the 28 g /kg DM inclusion of $NaNO_3$ significantly decreased total SCFA compared to the control but did not differ from the control total SCFA at 24 h. On the contrary, 7 and 14 g/kg DM inclusion levels of $NaNO_3$ significantly increased total SCFA at 24 h. At 12 and 24 h, acetate significantly increased in a linear manner with the levels of $NaNO_3$ inclusion and propionate tended to increase linearly. Butyrate significantly decreased in a linear manner throughout the incubation. Valerate followed a similar pattern of total SCFA, wherein the 28 g /kg DM inclusion level of $NaNO_3$ significantly decreased its concentration. A:P significantly increased with the 28 g /kg DM inclusion level of $NaNO_3$ following a linear trend.

The level effect of $NaNO_3$ inclusion with the MF diet tended to linearly affect total SCFA at 6 and 24 h of incubation and significantly affected total SCFA quadratically at 12 h (table 1.7). Total SCFA tended to decrease with the 28 g /kg DM inclusion level of $NaNO_3$ compared to the control at 6 h whereas the same inclusion level did not differ from the control at 12 and 24 h. Acetate increased linearly with the level of $NaNO_3$ inclusion at 12 and 24 h, wherein the 28 g /kg DM inclusion of $NaNO_3$ significantly increased acetate compared to the control. Propionate was affected by $NaNO_3$ treatment in a quadratic tendency. The 28 g /kg DM $NaNO_3$ inclusion level did not differ from the control while it tended to increase compared to 7-g/kg DM inclusion.

Butyrate significantly decreased with the levels of $NaNO_3$ inclusion linearly at 6 and 12 h and quadratically at 24 h. Valerate significantly decreased linearly with the $NaNO_3$ inclusion levels while significantly increasing linearly at 24 h. A:P tended to increase linearly with the levels of $NaNO_3$ inclusion at 12 h.

The level effect of $NaNO_3$ inclusion on total SCFA was significant towards a linear decline at 6 h and a quadratic decline at 12 h (table 1.8). There was not treatment effect on total SCFA by $NaNO_3$ inclusion at 24 h. Acetate significantly increased with the levels of $NaNO_3$ inclusion linearly at 6 h and quadratically at 12 h. Propionate significantly decreased linearly at 6 h and tended to decrease quadratically at 12 h. Butyrate significantly decreased linearly at 6 h and quadratically at 12 and 24 h. No treatment effects of $NaNO_3$ inclusion were observed for isobutyrate throughout the incubation period. At 12 h, a significant, quadratic decrease was observed for valerate.

Effects of $NaSO_4$ on CH_4, pH, and NH_3-N

Tables 1.9-1.11 summarize the data for the effect of $NaSO_4$ on CH_4, pH, and NH_3-N from in the HF, MF, and LF diets, respectively. CH_4 was unaffected by the addition of $NaSO_4$ compared to the control in all diets except for the MF diet, wherein CH_4 linearly increased with the inclusion levels (table 1.10). Similarly, pH and NH_3-N were largely unaffected by the $NaSO_4$ treatment (tables 1.9-1.11); however, there was a linear increase of pH along with the inclusion levels in the HF diet at 24 h and that of NH_3-N at 12 h in the same diet.

Effects of $NaSO_4$ on SCFAs as In-Vitro Fermentation Products

The data for the effect of $NaSO_4$ on SCFAs are summarized in tables 1.12-1.14 for the HF, MF, and LF diets, respectively. Similar to the above effects of $NaSO_4$ on CH_4, pH, and NH_3-N, total and individual SCFAs were largely unaffected by $NaSO_4$ on all diets (tables. 1.12-1.14).

In the HF diet at 24 h, total SCFA tended to increase linearly, accompanied with a significant quadratic increase in acetate and a significant linear decrease in butyrate (table 1.12). In the MF diet, propionate significantly decreased in a linear manner with the inclusion levels of $NaSO_4$ at 24 h, which caused the A:P to increase in the same fashion (table 1.13). On the other hand, A:P quadratically decreased in the LF diet, but acetate and propionate only numerically decreased and increased, respectively (table 1.14).

Effects of 3NPA on CH_4, pH, and NH_3-N

Tables 1.15-1.17 show the summary of CH_4, pH, and NH_3-N data for the effect of 3NPA in the HF, MF, and LF diets, respectively. In the 3NPA treatment compared to the control, the inclusion levels reduced CH_4 at 6 h of incubation linearly with the HF and LF diets and quadratically with the MF diet (tables 1.15-1.17). At the 2.0 g/kg DM inclusion level of 3NPA, CH_4 was reduced to 54, 38, and 40% of the control value in the HF, MF, and LF diets, respectively. Mitigation of CH_4 by 3NPA seems to have sustained at 12 h only for the HF and MF, wherein treatment tended to decrease CH_4 quadratically and linearly, respectively. CH_4 did not differ at the 24 h of incubation. The effect of 3NPA on pH was largely inconsistent. In the HF diet, pH tended to decrease linearly at 6 h, significantly decreased at 12 h, and tended to increase at 24 h peaking at the 0.5 g/kg DM 3NPA inclusion level (table 1.15). In the MF and LF diets, 3NPA significantly increased pH only at 12 h of incubation. Changes in NH_3-N by the 3NPA treatment was not statistically significant (tables 1.15-1.17).

Effects of 3NPA on SCFAs as In-Vitro Fermentation Products

SCFA data from the 3NPA treatment are summarized in tables 1.18-1.20 for the HF, MF, and LF diets, respectively. Compared to the control, 3NPA had little and inconsistent effects on total and individual SCFAs (tables 1.18-1.20). In the HF diet at 6 h, acetate and propionate

linearly decreased and increased, respectively, causing the A:P to decrease linearly (table 1.18). Butyrate tended to increase while valerate significantly increased at 12 h, whereas there was no significant effect at 24 h in the HF diet (table 1.18). In the MF diet, total SCFA tended to quadratically increase at 12 h, wherein butyrate significantly increased, and acetate tended to decrease, both linearly (table 1.19). At 24 h, total SCFA significantly decreased with the 3NPA inclusion levels (table 1.19). Lastly in the LF diet, total SCFA significantly decreased linearly at 6 h, acetate significantly increased, and butyrate valerate and isovalerate significantly decreased, compared to the control (table 1.20). There was no significant effect at 12 h, and acetate significantly increased quadratically compared to the control (table 1.20).

1.5 Discussion

Potency of Nitrate as an Anti-Methanogenic Agent

Nitrate (NO_3^-) has long been investigated for its anti-methanogenic potential and the toxicity of its intermediate, nitrite (NO_2^-), to the ruminant as well as to some of its microbes including methanogens (Rimington & Quin, 1933; Winter, 1967; Iwamoto et al., 2002). The reduction of NO_3^- in the rumen comprises two steps collectively known as dissimilatory nitrate reduction to ammonia (DNRA): first, NO_3^- to NO_2^- , and then NO_2^- to ammonia (NH_3) or ammonium (NH_4^+) consuming a total of 8 electrons (Thauer et al., 1977). In the present study, CH_4 was reduced to 91% of the control value with the $NaNO_3$ addition of 28 g/kg DM when averaged across the diets (tables 1.3-5; figure 1.2). There are possible two mechanisms, by which $NaNO_3$ decreased CH_4.

First, DNRA competes with methanogenesis for H_2, thus inhibiting the production of CH_4. DNRA is thermodynamically more favorable ($-\Delta G^o$ = 599.6 kJ/mol) than methanogenesis through reduction of CO_2 ($-\Delta G^o$ = 131 kJ/mol) and redirects the flow of H_2 away from the

reduction of CO_2 to CH_4 (Thauer et al., 1977). Each reduction step of NO_3^- ($-\Delta G^o$ = 163.2 kJ/mol) and NO_2^- ($-\Delta G^o$ = 436.4 kJ/mol) is also thermodynamically favored in comparison to methanogenesis (Thauer et al, 1977).

Second, methanogens are susceptible to NO_2^- toxicity. The NO_2^- toxicity on methanogens was demonstrated in a study by Iwamoto et al. (2002), wherein CH_4 production was inhibited by 50 % when 0.5 mM of NO_2^- was added to a mixed methanogen culture. Further, CH_4 was inhibited by the addition of 5 mM NO_3^- in a co-culture of mixed methanogens with one of the following nitrate reducers: *Selenomonas ruminantium* subsp. *lactilytica* TH1, *Veillonella parvula*, or *Wolinella succinogenes*, suggesting that the accumulation of NO_2^- was detrimental to methanogens (Iwamoto et al., 2002).

Although it is unknown whether H_2 competition or NO_2^- toxicity is the primary mode of action when addition of NO_3^- inhibits CH_4, recent studies on this topic suggest that the NO_2^- toxicity may play a larger role in the anti-methanogenic effect of NO_3^- supplementation than previously thought (Iwamoto et al., 2002; Lee et al., 2017; van Lingen et al., 2021). The above three bacterial species – *S. ruminantium* subsp. *lactilytica* TH1, *V. parvula*, and *W. succinogenes* – are capable of NO_3^- reduction as well as reduction of other electron acceptors such as fumarate and formate as a means of respiration (Stewart et al, 1996; Kröger et al., 2002). In the present study, calculation of NO_3^--N recovery as NH_3-N shows above 100% for the 28 g/kg DM inclusion level of $NaNO_3$ at 24 h of incubation (data not shown). Therefore, although microbial composition in the samples were not analyzed in the present study, it is likely that some if not all of these bacteria reduced NO_3^- and decreased CH_4. Additionally, the above 100% N-recovery from $NaNO_3$ addition in the present study may be attributed to the accumulation of NH_3-N (figure 1.4) as a result of metabolism of microbial carcass protein by other microbes (Chen &

Russell, 1989; Bach et al., 2005). Figure 1.3 shows an increase of NH_3-N compared to the control in the $NaNO_3$ treatment in the LF diet at 24 h, the difference was not significant, perhaps, because starch-rich diets such as the LF in this study can increase the population of NH_3-N assimilating bacteria (Phillipson et al., 1959). Further, requirement of minimum NH_3-N concentration to support maximum microbial growth is higher for substrates that require extensive digestion than for those easily digested (Odle & Schaefer, 1987).

Nonetheless, it remains unclear whether the supplied NO_3^- was completely reduced to NH_3 in this study. In a monoculture, these nitrate-reducing bacteria were capable of growth only when 5 mM NO_3^- and 40 mmol/L H_2 were added to a medium containing 30 mM succinate as a carbon source whereas only *S. ruminantium* subsp. *lactilytica* TH1 and *W. succinogenes* grew even when the NO_3^- in the medium was switched to NO_2^- (Iwamoto et al., 2002). These results demonstrate that *S. ruminantium* subsp. *lactilytica* TH1 and *W. succinogenes* both possess electron transport phosphorylation (ETP) systems coupled with nitrate and nitrite reductases and *V. parvula* only with nitrate reductase (Iwamoto et al., 2002). With the prevention of NO_2^- accumulation with respect to animal health, it is likely desirable to increase the number of *W. succinogenes* with $NaNO_3$ treatment because this bacterium has the greatest reduction rate of nitrate and nitrite among the three known nitrate-reducers (Iwamoto et al., 2002).

As described above, *W. succinogenes* and *S. ruminantium* subsp. *lactilytica* are capable of NO_2^- reduction to NH_3 because they possess NO_2^- reductase (Nrf) as well as NO_3^- reductase (Nap) (Wang et al., 2019). The Nrf/Nap complex operates under a relatively low NO_3^- concentration of 5 mM or below and conducts DNRA in the periplasm (figure 1.5A & B) (Wang et al., 2019). In case of NO_3^- concentrations above 5 mM, some bacteria such as *S. ruminantium* subsp. *lactilytica* (Yoshii et al., 2003; Kaneko et al., 2015) and non-ruminal strain of *Escherichia*

coli (Wang et al., 2019) can utilize the cytoplasmic NO_3^- and NO_2^- reductases, Nar and Nir, respectively (Wang et al., 2019; figure 1.5B). In the present study, the 28 g/kg DM level of $NaNO_3$ provides the NO_3^- concentration of 11 mM by itself. Therefore, it is possible that the Nar/Nir complex of *S. ruminantium* subsp. *lactilytica* may have largely contributed to DNRA, which would increase the number of *S. ruminantium* subsp. *lactilytica* in samples with high NO_3^- concentration. This would also support the drastic decreases in CH_4 and high N-recovery rate with the high inclusion level of $NaNO_3$ through H_2 competition. Another scenario is that NO_2^- could have accumulated after NO_3^- reduction, which would then be toxic to methanogens as well as other microorganisms. This latter scenario may as well explain both CH_4 reduction and N-recovery rate. Microbial profile of these samples with low CH_4 and high NH_3-N may further help clarify the effect of $NaNO_3$ treatment.

Additionally, the elevated pH in the $NaNO_3$ treatment group compared to the control in the present study suggests that organic acids such as SCFAs and lactate did not accumulate to the point of acidosis (Aikman et al., 2011). Indeed, lactate was below detectable levels, and lactate-utilizing bacteria such as *Megasphaera elsdenii* and *S. ruminantium* subsp. *lactilytica* are naturally present in the rumen (Aikman et al., 2011). This way, the increase in pH may also support possible proliferation of *S. ruminantium* subsp. *lactilytica* as well as that of *M. elsdenii* especially when 28 g/kg $NaNO_3$ was added to the culture regardless of the diet (figure 1.3).

Other possible causes for the changes in pH are changes in concentration of strong ions such as Na^+ and pressure of CO_2 in the gas phase (Kohn & Dunlap, 1998). Even though NO_3^- was delivered as $NaNO_3$, the effect of Na^+ was miniscule because a significant increase in pH was hardly observed in the $NaSO_4$ treatment group compared to the $NaNO_3$ treatment (figure

1.3). Thus, the increases of pH in the present study were most likely influenced by both the pressure of CO_2 and SCFA composition arising from altered fermentation pattern.

The comparison of pH between the control and $NaNO_3$ allows for speculation of the CO_2 pressure in the *in-vitro* bottles. In $NaNO_3$ treatment samples, pH significantly increased in most diet and hours of incubation (figure 1.3). pH and pressure of CO_2 in batch cultures has an inverse relationship expressed as: $pH = 7.74 + \log([HCO_3^-]/pCO_2)$, since CO_2 establishes an equilibrium between gaseous and liquid phases of the rumen and *in-vitro* system due to their proximity (Kohn & Dunlap, 1998). Therefore, the rise in pH may be associated with a decrease in CO_2 gas in the headspace of the fermentation bottles.

Aside from its conversion to $HCO3^-$ and reduction to CH_4, CO_2 is a substrate for acetate (Hackmann et al, 2017), whose increase shows a significant correlation ($R = 0.72$, $p < 2.2e\text{-}16$) with pH in the present study (figure 1.6). Even though the metabolic cause and fate for decreased CO_2 was not directly measured, there is a clear influence of pH on increased acetate. Typically, ruminal SCFA production depends on the F:C of the diet, and high concentrate diets decrease pH and A:P for instance, compared to high forage diets (Russell, 1998). Within each diet of the present study, A:P was not affected by the $NaNO_3$ addition at 24 h of incubation but differed significantly between the HF and LF diets ($A:P_{HF} > A:P_{LF}$) and between the MF and LF diets ($A:P_{MF} > A:P_{LF}$) when compared across the diets with the 28 g/kg DM $NaNO_3$ inclusion (data not shown). Treatment effect of $NaNO_3$ was not observed on A:P for all diets at 24 h of incubation (tables 1.6-1.8). Further analysis of the data revealed that, in accordance with the relationship between A:P and pH described above, F:C in the diet significantly affected pH and followed the order: $pH_{HF} > pH_{MF} > pH_{LF}$ (tables 1.3-1.5). pH and A:P also showed a significant correlation ($R = 0.73$, $p < 2.2e\text{-}16$; figure 1.7).

Interestingly, treatment effect on acetate within each diet was observed only for the HF and MF diets with the 28 g/kg DM $NaNO_3$ inclusion, which is not reflected in the A:P (tables 1.6 & 1.7). However, in these samples, propionate also tended to increase, explaining why A:P was unaffected by the $NaNO_3$ treatment (tables 1.6 & 1.7). Propionate production occurs through acrylate and succinate pathways, where lactate and succinate are the major entry substrate, respectively (Hackmann et al., 2017). Lactate is converted to propionate by *V. parvula* and *M. elsdenii* in the acrylate pathway and by *Veillonella alcalescens* and *S*. *ruminantium* in the succinate pathway (Ng & Hamilton, 1971; Johns, 1951; Baldwin et al., 1965; Paynter & Elsden, 1970; Hino & Kuroda, 1993) although *V. parvula* has been shown to convert succinate to propionate only in the presence of lactate (Janssen, 1992). In fact, acetate production from lactate is disproportionately preferred to propionate production from lactate by *V. parvula* while malate is proportionately converted to acetate and propionate (Denger & Schink, 1992). V. parvula preferentially produces acetate from lactate than propionate while converting succinate solely to propionate, indicating a fermentation shift towards acetate production in the presence of lactate (Denger & Schink, 1992).

On the other hand, *S. ruminantium* subsp. *lactilytica* ferments glucose to lactate, propionate, and acetate and lactate to propionate and acetate in a decreasing order of relative amounts produced (Kanegasaki & Takahashi, 1967). In contrast to the speculation of *S. ruminantium* subsp. *lactilytica* proliferation from pH and NH_3-N, acetate and propionate in the present study follows the lactate metabolism pattern of *V. parvula*. However, it is also possible that *S. ruminantium* subsp. *lactilytica* in abundance may have contributed to glucose fermentation to lactate, which was then converted to acetate and propionate by *V. parvula* because *S. ruminantium* subsp. *lactilytica* predominantly ferment glucose to lactate during its

rapid growth (Melville et al., 1988) and *V. parvula* is generally present at a lower abundance than *S. ruminantium* subsp. *lactilytica* (Latham et al., 2016).

Despite the increase in acetate and propionate, total SCFA in the 28 g/kg DM $NaNO_3$ treatment was not significantly different from the control in all diets since butyrate significantly decreased linearly in the HF diet (table 1.6) and quadratically in the MF and LF diets (tables 1.7 & 1.8). At similar NO_3^- inclusion levels, decrease in butyrate was not observed in an *in-vitro* study by Van Zijderveld et al. (2010), *in-vivo* studies by Hulshof et al. (2012), de Raphélis-Soissan et al. (2014), Veneman et al. (2015), and Olijhoek et al. (2016). However, observation *in vivo* similar to the present study was described by Farra and Satter (1971) for acetate and butyrate while total SCFAs, valerate, and iso-SCFAs (isobutyrate and isovalerate) were not consistently affected.

Potential of $NaSO_4$ for CH_4 Reduction

Similar to NO_3^-, sulfate (SO_4^{2-}) is an electron acceptor and can be reduced to hydrogen sulfide (H_2S); this exothermic reaction is more thermodynamically favorable ($-\Delta G^o$ = 234 kJ/mol; Ungerfeld & Kohn, 2006) than methanogenesis ($-\Delta G^o$ = 131 kJ/mol; Thauer et al., 1977). However, the present study did not reflect this theory (tables 1.9-1.11). In a study by van Zijderveld et al. (2010), 26 g/kg DM SO_4^{2-} reduced CH_4 significantly but only by 15% *in-vivo*. In contrast, CH_4 was significantly increased by 32% with the 12 g/kg $NaSO_4$ inclusion level in the MF diet (table 1.10). In another study, SO_4^{2-} did not compete with methanogenesis for H_2 at inclusion levels lower than 20 mM SO_4^{2-} (Ohashi et al., 1996). The estimates of SO_4^{2-} concentration in our study were 2.8 mM for the 12 g/kg $NaSO_4$ treatment. This is consistent with the result from Gupta et al. (2017), where 2.3 mM of $NaSO_4$ did not inhibit CH_4. The amount of $NaSO_4$ used in the present study was, therefore, too low to inhibit CH_4. The effect of $NaSO_4$

treatment effects on pH and NH_3-N are similar to those reported by Zhao et al. (2020), especially for the LF diet (table 1.11). SO_4^{2-} treatment seems to pose inconclusive effects on total and individual SCFA (van Zijderveld et al., 2010; Gupta et al., 2017; Zhao et al., 2020).

Overall, $NaSO_4$ treatment in this study did not meaningfully affect *in-vitro* methanogenesis and fermentation pattern. Several studies suggest that sulfate-reducing bacteria (SRB) compete with methanogens for H_2 poorly at low levels (Ohashi et al., 1996; Isa et al., 1986; van Zijderveld et al., 2010). Higher inclusion levels (> 26 g/kg DM $NaSO_4$) may be of interest for future research on anti-methanogenic potentials of SO_4^{2-}. Caution needs to be taken, however, when the total sulfur (S) content exceeds 4g/kg DM for the risk of polioencephalomalacia to the ruminants from re-inhaled H_2S (van Zijdeveld et al., 2010; Gould, 1998). Nevertheless, H_2S is readily available to sulfide-assimilating ruminal microbes for further fermentation (Kandylis, 1984; Gupta et al., 2017), and van Zijderveld et al. (2010) did not observe any clinical signs of polioencephalomalacia with an *in-vivo* use of 26 g/kg DM of SO_4^{2-}.

Anti-methanogenic potential of 3NPA

3NPA occurs in leguminous plants, namely of the genera *Astragalus*, and is considered a toxin (Williams et al., 1976). After it was proposed that 3NPA is metabolized to NO_2^- and propionate via the cleavage of the nitro group from the aliphatic nitro compound (Majak & Cheng, 1981), Anderson et al. (1993) suggested that the nitro group of 3NPA is reduced instead to form β-alanine, thus serving as a H_2 sink and a potential anti-methanogenic agent. In the present study, CH_4 moderately decreased with the 3NPA treatment especially at the early hours of incubation (figure 1.8).

However, the depressed CH_4 was concomitant with a decrease in pH (figure 1.9), and Pearson analysis shows a negative correlation (R = -0.75, p < 2.2e-16) between the two response

variables (figure 1.10). Anderson and Rasmussen (1998) speculated that the anti-methanogenic mode of action of 3NPA may be its toxicity to methanogens, rather than its property as a potential H_2 sink because CH_4 reduction was independent of metabolized 3NPA. The decline in pH in the present study may suggest the toxicity not only to methanogens but also to other fermentative bacteria, as it may reflect accumulation of organic acids such as lactate and SCFAs (Aikman et al., 2011).

Although the mechanism of 3NPA toxicity to ruminal microbes is unclear, there remains a possible metabolism of 3NPA to propionate and NO_2^-, as originally proposed by Majak and Cheng (1981). Perhaps, conversion of 3NPA to propionate is a minor effect producing less than 10% of 3NPA stoichiometrically (Anderson et al., 1993). In the present study, propionate was largely unaffected, except for a linear increase by the levels of 3NPA inclusion at 6 h in the HF diet (tables 1.18-1.20). On the other hand, 3NPA metabolism to NO_2^- may exert a greater influence on the toxicity of 3NPA; however, little evidence is reported for the accumulation of NO_2^- upon 3NPA inclusion (Majak & Cheng, 1981; Anderson et al., 1993). However, reduction of NO_2^- to ammonia may explain the lack of NO_2^- accumulation (Majak & Cheng, 1981; Anderson et al., 1993). In the present study, NH_3-N significantly increased at 6 h as CH_4 significantly decreased in the LF diet (table 1.17). Though NO_2^- was not directly measured in the present study, the accumulation of NH_3-N at 6 h when CH_4 decreased the most, may provide a support for the nitro-cleavage pathway of 3NPA.

As expected, Pearson analysis revealed a negative correlation (R = -0.92, p < 2.2e-16) between pH and total SCFA in the four inclusion levels of 3NPA (figure 1.11). However, lactate was not measurable in the present study. Further, a decrease of the A:P in the HF diet was

observed at 6 h, which is consistent with the finding by Anderson and Rasmussen (1998). The fermentation pattern was slightly altered by 3NPA, which may be more due to its toxicity.

Conclusion

In conclusion, the addition of $NaNO_3$ clearly mitigated methanogenesis and altered the fermentation pattern via decreases in CH_4 and butyrate and increases in pH, NH_3-N, acetate, and propionate. The addition of $NaSO_4$ produced inconsistent results, perhaps due to lower inclusion levels in the present study than necessary to exert its anti-methanogenic effect. The effects of 3NPA inclusion on methanogenesis and fermentation parameters were observed at early hours of incubation and may demonstrate the co-existence of two possible metabolic pathways for the compound as well as 3NPA and NO_2^- toxicity. Since microorganisms are the major component of feed digestion in the rumen, analysis of microbial composition would be necessary to clarify the effects of feed additives on gas production and fermentation pattern in the present study. However, this study demonstrated the potency of $NaNO_3$ as an anti-methanogenic agent, which is expected to shift the microbial community in the rumen fluid. The anti-methanogenic mechanism of NO_3^- remains elusive despite decades of research and needs elucidating. The present study also sheds light to the role of thermodynamics in the rumen environment, where theoretically competitive reactions may or may not actually predominate, e.g., DNRA or SO_4^{2-} reduction *vs.* methanogenesis.

1.6 Tables

Table 1.1. Formulation of three basal diets (high, medium, and low forage) in a 500 g batch used for the experiments

Ingredients	Basal diets		
	High forage (F:C=70:30)	Medium forage (50:50)	Low forage (30:70)
Alfalfa pellets (F), *g*	350	250	150
Concentrate mix[1] (C), *g*	150	250	350
Ground corn, *g*	119.8	204.4	288.7
Soybean meal, *g*	25.2	40.6	56.3
Vitamin & mineral premix[2], *g*	5.0	5.0	5.0
Zn, *g*	0.06	0.06	0.06
Cu, *mg*	16.9	16.9	16.9
Co, *mg*	0.13	0.13	0.13
I, *mg*	1.30	1.30	1.30
Fe, *mg*	6.50	6.50	6.50
Mn, *mg*	39.0	39.0	39.0
Se, *mg*	0.39	0.39	0.39
Vitamin A, *IU*	8267	8267	8267
Vitamin D_3, *IU*	1102	1102	1102
Vitamin E, *IU*	5.51	5.51	5.51
Total, *g*	500	500	500

[1]Concentrate mix included ground corn, soybean meal, and vitamin and mineral mix.
[2]Vitamin and mineral mix included 1.2% Zn, 3,380 ppm Cu, 26 ppm Co, 260 ppm I, 1,300 ppm Fe, 7,800 ppm Mn, 78 ppm Se, 750,000 IU/lb vitamin A, 100,000 IU/lb vitamin D_3, 5,000 IU/lb vitamin E.

Table 1.2. Chemical composition of three basal diets (high, medium, and low forage) on a dry matter basis (DM)

Chemical composition[1]	Basal diets		
	High Forage (F:C=70:30)	Medium Forage (50:50)	Low Forage (30:70)
Dry matter, *%*	88.8	88.4	88.0
Acid detergent fiber, *%*	27.1	20.7	14.2
Neutral detergent fiber, *%*	35.9	28.8	21.7
Crude protein[2], *%*	18.0	18.1	18.2
Metabolizable energy, *Mcal/kg DM*	2.28	2.35	2.42
Net energy, *Mcal/kg DM*	1.72	1.93	2.15
Ether extract, *%*	3.16	3.46	3.75
Ca, *%*	1.2	0.88	0.57
P, *%*	0.34	0.36	0.38
Mg, *%*	0.28	0.25	0.21
K, *%*	2.10	1.80	1.40
Na, *‰*	0.86	0.68	0.51
Cl, *%*	0.54	0.42	0.29
S, *%*	0.25	0.23	0.20

[1]Chemical composition of each basal diet was calculated from nutrient values obtained in NRC (2001) for alfalfa hay (International Feed #: 1-00-023), ground corn (4-02-854), and soybean meal (5-20-638) unless otherwise stated.
[2]Crude protein contents of ground corn and soybean meal were 16 and 48% DM, respectively.

Table 1.3. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, $p <$	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.34[a]	0.76[b]	0.28[c]	0.00[c]	0.12	0.01	0.01
pH	5.87[a]	6.38[b]	6.02[ab]	6.73[bc]	0.12	0.01	0.48
NH_3-N, *mg/dL*	8.80[d]	11.72[de]	14.42[e]	8.94[d]	1.87	0.92	0.02
12 hours							
CH_4, *mM*	1.99[a]	1.55[ab]	0.91[bc]	0.15[c]	0.41	0.01	0.70
pH	5.54[a]	5.67[a]	5.72[a]	5.92[b]	0.06	0.01	0.96
NH_3-N, *mg/dL*	8.22[a]	11.01[b]	10.80[b]	12.43[b]	0.54	0.01	0.14
24 hours							
CH_4, *mM*	1.67[a]	1.72[a]	1.02[b]	0.06[c]	0.24	0.01	0.16
pH	5.21[a]	5.30[b]	5.34[b]	5.57[c]	0.02	0.01	0.13
NH_3-N, *mg/dL*	8.92[d]	9.36[d]	9.50[d]	14.08[e]	2.84	0.02	0.26

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.4. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, $p <$	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.20[a]	0.81[b]	0.40[c]	0.14[c]	0.11	0.01	0.05
pH	5.82[e]	6.19[f]	6.21[f]	6.09[f]	0.10	0.18	0.03
NH_3-N, *mg/dL*	9.48	12.12	10.11	12.12	1.81	0.34	0.89
12 hours							
CH_4, *mM*	2.29[a]	1.73[ab]	1.28[b]	0.15[c]	0.38	0.01	0.89
pH	5.52[a]	5.53[a]	5.68[b]	5.94[c]	0.05	0.01	0.15
NH_3-N, *mg/dL*	11.18	11.10	13.40	11.38	2.10	0.86	0.54
24 hours							
CH_4, *mM*	1.20[a]	0.80[b]	0.40[c]	0.14[c]	0.11	0.01	0.05
pH	5.21[a]	5.25[b]	5.28[c]	5.52[d]	0.02	0.01	0.01
NH_3-N, *mg/dL*	8.41[a]	8.62[a]	8.82[a]	12.86[b]	2.69	0.01	0.19

[a-d]different superscripts within a row differ ($p \leq 0.05$)
[e, f]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.5. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, $p <$	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.49[a]	1.20[b]	0.33[c]	0.19[d]	0.05	0.01	0.01
pH	5.87[a]	5.93[b]	6.15[c]	6.16[c]	0.02	0.01	0.01
NH_3-N, *mg/dL*	8.19	9.28	12.64	13.64	1.65	0.07	0.56
12 hours							
CH_4, *mM*	1.92[a]	1.68[a]	1.22[b]	0.14[c]	0.14	0.01	0.13
pH	5.50[a]	5.52[a]	5.50[a]	5.90[b]	0.04	0.01	0.01
NH_3-N, *mg/dL*	7.57[a]	8.66[a]	8.89[a]	11.92[b]	2.30	0.01	0.46
24 hours							
CH_4, *mM*	2.50[a]	2.17[a]	1.54[b]	0.24[c]	0.40	0.01	0.37
pH	5.12[a]	5.18[b]	5.23[b]	5.40[c]	0.02	0.01	0.16
NH_3-N, *mg/dL*	8.29	8.32	9.08	11.36	2.66	0.03	0.48

[a-d]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Figure 1.6. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, $p <$	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.07	106.44	99.90	73.01	11.81	0.07	0.30
Individual SCFA	*mol %*						
Acetate (A)	64.27	66.41	66.72	65.01	1.58	0.89	0.24
Propionate (P)	23.98	22.58	23.22	24.78	1.34	0.54	0.37
Butyrate	9.68[a]	9.06[a]	8.21[b]	7.78[c]	0.23	0.01	0.14
Isobutyrate	0.44	0.43	0.43	0.60	0.08	0.15	0.35
Valerate	0.96	0.87	0.77	0.92	0.11	0.83	0.26
Isovalerate	0.68	0.65	0.64	0.92	0.10	0.12	0.24
A:P Ratio	2.69	2.97	2.88	2.72	0.21	0.90	0.35
12 hours							
Total SCFA, *mM*	128.86[a]	130.49[a]	130.21[a]	113.90[b]	8.13	0.01	0.06
Individual SCFA	*mol %*						
Acetate (A)	61.79[a]	62.86[ab]	63.90[b]	67.33[c]	1.24	0.01	0.28
Propionate (P)	26.01[d]	24.58[de]	24.67[de]	23.33[e]	1.69	0.02	0.72
Butyrate	10.23[a]	10.53[a]	9.44[b]	7.58[c]	0.57	0.01	0.13
Isobutyrate	0.41	0.41	0.44	0.42	0.04	0.49	0.52
Valerate	1.00[a]	1.09[a]	1.02[a]	0.74[b]	0.10	0.01	0.04
Isovalerate	0.55	0.52	0.53	0.59	0.07	0.41	0.38
A:P Ratio	2.45[a]	2.60[a]	2.63[a]	2.90[b]	0.22	0.01	0.85
24 hours							
Total SCFA, *mM*	152.92[a]	165.48[b]	166.26[b]	153.13[a]	2.72	0.50	0.01
Individual SCFA	*mol %*						
Acetate (A)	57.95[a]	59.39[a]	58.80[a]	61.36[b]	0.91	0.01	0.50
Propionate (P)	26.06[de]	25.12[d]	26.55[de]	27.12[e]	0.83	0.04	0.40
Butyrate	14.07[a]	13.52[ab]	12.59[b]	9.70[c]	0.36	0.01	0.11
Isobutyrate	0.32	0.35	0.43	0.35	0.04	0.46	0.06
Valerate	1.65	1.91	1.84	1.52	0.27	0.09	0.11
Isovalerate	0.52[de]	0.46[d]	0.53[e]	0.48[de]	0.05	0.31	0.87
A:P Ratio	2.23	2.37	2.23	2.28	0.09	0.98	0.62

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.7. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, $p <$	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.85^{d}	108.19^{d}	99.98^{de}	86.48^{e}	3.24	0.02	0.14
Individual SCFA	*mol %*						
Acetate (A)	62.86	64.75	65.74	67.31	1.10	0.06	0.58
Propionate (P)	24.78	23.84	23.02	22.05	1.18	0.16	0.77
Butyrate	10.24^{a}	9.20^{b}	9.00^{bc}	8.45^{c}	0.14	0.01	0.06
Isobutyrate	0.40	0.53	0.50	0.54	0.04	0.14	0.34
Valerate	1.05^{a}	0.95^{b}	0.97^{b}	0.83^{c}	0.03	0.01	0.89
Isovalerate	0.66^{d}	0.75^{de}	0.81^{e}	0.83^{d}	0.03	0.04	0.22
A:P Ratio	2.56	2.72	2.86	3.05	0.16	0.11	0.78
12 hours							
Total SCFA, *mM*	118.34^{ab}	126.74^{a}	129.58^{a}	107.47^{b}	4.77	0.05	0.01
Individual SCFA	*mol %*						
Acetate (A)	60.48^{a}	60.83^{a}	61.97^{a}	66.22^{b}	1.20	0.01	0.14
Propionate (P)	25.74	26.54	24.97	23.42	2.03	0.11	0.56
Butyrate	11.75^{a}	10.95^{a}	11.19^{a}	8.48^{b}	0.92	0.01	0.21
Isobutyrate	0.37^{ab}	0.31^{a}	0.36^{ab}	0.43^{b}	0.04	0.03	0.06
Valerate	1.13^{d}	0.92^{de}	1.04^{de}	0.83^{e}	0.10	0.03	1.00
Isovalerate	0.52^{a}	0.45^{b}	0.46^{ab}	0.63^{c}	0.05	0.01	0.01
A:P Ratio	2.39^{d}	2.42^{d}	2.52^{de}	2.84^{e}	0.22	0.01	0.43
24 hours							
Total SCFA, *mM*	156.83^{de}	159.14^{d}	157.30^{d}	146.93^{e}	4.66	0.04	0.16
Individual SCFA	*mol %*						
Acetate (A)	55.26^{a}	55.86^{a}	55.97^{a}	58.24^{b}	0.63	0.01	0.19
Propionate (P)	26.83^{de}	25.68^{d}	26.20^{de}	27.48^{e}	1.03	0.14	0.05
Butyrate	16.05^{a}	16.60^{a}	15.93^{a}	12.29^{b}	0.67	0.01	0.01
Isobutyrate	0.34	0.35	0.33	0.32	0.03	0.21	0.68
Valerate	1.00^{d}	1.01^{d}	1.08^{de}	1.18^{e}	0.08	0.01	0.67
Isovalerate	0.51	0.50	0.49	0.48	0.05	0.32	0.68
A:P Ratio	2.06	2.19	2.16	2.13	0.10	0.61	0.12

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.8. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaNO_3$, *g/kg DM*					Trend, *p* <	
	0	7	14	28	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	97.32[a]	97.10[a]	86.60[b]	77.91[b]	2.01	0.01	0.71
Individual SCFA	*mol %*						
Acetate (A)	60.56[a]	61.25[a]	65.70[b]	68.10[c]	0.52	0.01	0.40
Propionate (P)	25.42[a]	25.57[a]	21.72[b]	19.84[b]	0.82	0.01	0.92
Butyrate	11.41[a]	10.73[ab]	10.16[bc]	9.44[c]	0.23	0.01	0.39
Isobutyrate	0.56	0.52	0.55	0.57	0.03	0.50	0.48
Valerate	1.21	1.11	1.00	1.06	0.06	0.22	0.18
Isovalerate	0.85	0.82	0.87	0.99	0.04	0.06	0.29
A:P Ratio	2.38[a]	2.41[b]	3.03[c]	3.43[c]	0.10	0.01	1.00
12 hours							
Total SCFA, *mM*	121.80[a]	117.94[ac]	130.48[b]	112.73[c]	4.53	0.03	0.01
Individual SCFA	*mol %*						
Acetate (A)	58.47[a]	59.10[a]	57.96[a]	65.47[b]	1.09	0.01	0.01
Propionate (P)	25.52[d]	25.04[de]	25.96[d]	23.92[e]	1.34	0.05	0.13
Butyrate	13.88[a]	13.87[a]	14.07[a]	8.80[b]	0.74	0.01	0.01
Isobutyrate	0.39	0.38	0.37	0.40	0.04	0.64	0.36
Valerate	1.16[a]	1.08[a]	1.12[a]	0.78[b]	0.04	0.01	0.01
Isovalerate	0.58[ab]	0.54[a]	0.52[a]	0.62[b]	0.05	0.10	0.01
A:P Ratio	2.32[a]	2.39[a]	2.26[a]	2.76[b]	0.17	0.01	0.02
24 hours							
Total SCFA, *mM*	147.76	150.86	158.71	150.09	4.66	0.66	0.13
Individual SCFA	*mol %*						
Acetate (A)	49.37	49.90	50.06	51.90	1.75	0.12	0.71
Propionate (P)	26.13	24.95	25.90	28.04	1.66	0.14	0.25
Butyrate	22.26[a]	22.74[a]	21.75[a]	17.69[b]	2.71	0.01	0.01
Isobutyrate	0.38	0.40	0.40	0.39	0.01	0.82	0.48
Valerate	1.23	1.35	1.25	1.33	0.14	0.28	0.74
Isovalerate	0.62	0.66	0.64	0.64	0.02	0.68	0.42
A:P Ratio	1.90	2.07	1.94	1.85	0.14	0.58	0.48

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.9. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, $p <$	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.34	1.33	1.33	1.22	0.10	0.24	0.60
pH	5.87	5.87	5.82	5.88	0.02	0.84	0.15
NH_3-N, *mg/dL*	8.80	8.71	8.08	10.32	1.51	0.48	0.48
12 hours							
CH_4, *mM*	1.99	2.13	2.08	1.87	0.44	0.34	0.23
pH	5.54	5.55	5.56	5.57	0.03	0.40	0.81
NH_3-N, *mg/dL*	8.22[a]	9.28[a]	13.05[b]	13.26[b]	1.12	0.01	0.22
24 hours							
CH_4, *mM*	1.67	1.47	1.62	1.82	0.44	0.38	0.37
pH	5.21[a]	5.26[b]	5.25[b]	5.28[b]	0.01	0.01	0.12
NH_3-N, *mg/dL*	8.92	8.70	10.30	10.89	2.77	0.18	0.98

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.10. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, $p <$	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.20	1.30	1.41	1.34	0.10	0.16	0.14
pH	5.82	5.88	5.84	5.87	0.02	0.18	0.69
NH_3-N, *mg/dL*	9.48	9.20	9.86	10.37	1.69	0.67	0.91
12 hours							
CH_4, *mM*	2.29	1.97	1.91	2.14	0.36	0.64	0.05
pH	5.52	5.42	5.53	5.50	0.04	0.40	0.38
NH_3-N, *mg/dL*	11.18	8.29	7.49	8.22	1.77	0.22	0.16
24 hours							
CH_4, *mM*	1.82[a]	2.16[b]	2.29[b]	2.40[b]	0.42	0.01	0.11
pH	5.21	5.20	5.19	5.19	0.01	0.31	0.48
NH_3-N, *mg/dL*	8.41	8.36	7.95	7.61	2.23	0.26	0.98

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.11. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, $p <$	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.49	1.53	1.46	1.46	0.07	0.65	0.99
pH	5.87	5.91	5.93	5.93	0.03	0.09	0.30
NH_3-N, *mg/dL*	6.91	7.07	9.28	8.72	1.94	0.40	0.61
12 hours							
CH_4, *mM*	1.92	1.53	1.72	1.95	0.25	0.67	0.36
pH	5.50[c]	5.38[d]	5.45[cd]	5.39[d]	0.03	0.10	0.44
NH_3-N, *mg/dL*	7.57	6.12	6.52	6.81	2.03	0.75	0.39
24 hours							
CH_4, *mM*	2.50	2.54	2.33	2.32	0.46	0.03	0.74
pH	5.12	5.10	5.11	5.13	0.02	0.53	0.32
NH_3-N, *mg/dL*	8.29[ab]	9.41[a]	7.10[ab]	5.50[b]	2.27	0.02	0.45

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.12. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, *p* <	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.07	104.96	101.53	102.14	9.50	0.94	0.95
Individual SCFA	*mol %*						
Acetate (A)	64.27	63.82	63.40	63.59	1.04	0.26	0.30
Propionate (P)	23.98	24.74	25.03	24.88	0.86	0.29	0.31
Butyrate	9.68	9.32	9.49	9.42	0.22	0.57	0.55
Isobutyrate	0.43	0.48	0.44	0.46	0.07	0.88	0.83
Valerate	0.96	0.96	0.97	0.97	0.06	0.93	0.96
Isovalerate	0.68	0.67	0.67	0.67	0.08	1.00	0.94
A:P Ratio	2.69	2.60	2.54	2.58	0.13	0.30	0.28
12 hours							
Total SCFA, *mM*	128.86	126.84	130.93	124.57	5.33	0.31	0.34
Individual SCFA	*mol %*						
Acetate (A)	61.79	62.38	62.06	62.18	1.44	0.62	0.55
Propionate (P)	26.01	25.33	25.32	25.17	1.81	0.18	0.38
Butyrate	10.23	10.27	10.55	10.57	0.44	0.17	0.69
Isobutyrate	0.41	0.39	0.40	0.40	0.04	0.81	0.77
Valerate	1.01[a]	1.14[b]	1.16[b]	1.15[b]	0.07	0.03	0.04
Isovalerate	0.55	0.50	0.52	0.53	0.05	0.77	0.23
A:P Ratio	2.45	2.50	2.50	2.50	0.23	0.41	0.46
24 hours							
Total SCFA, *mM*	152.92[c]	163.33[d]	159.25[cd]	165.97[d]	2.92	0.02	0.47
Individual SCFA	*mol %*						
Acetate (A)	57.95[a]	59.32[b]	59.24[b]	59.33[b]	1.14	0.02	0.04
Propionate (P)	26.06	26.18	25.62	26.04	1.03	0.79	0.43
Butyrate	14.07[a]	12.57[b]	13.19[ab]	12.79[b]	0.55	0.05	0.10
Isobutyrate	0.32	0.39	0.39	0.36	0.04	0.45	0.09
Valerate	1.08[c]	1.01[cd]	1.03[cd]	0.97[d]	0.03	0.03	0.75
Isovalerate	0.52	0.54	0.54	0.50	0.06	0.25	0.18
A:P Ratio	2.23	2.28	2.32	2.28	0.10	0.26	0.14

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.13. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, *p* <	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.85	102.47	104.43	102.02	2.67	0.89	0.67
Individual SCFA	*mol %*						
Acetate (A)	62.86	63.67	62.90	62.67	1.17	0.58	0.56
Propionate (P)	24.78	25.48	26.25	26.09	1.04	0.42	0.57
Butyrate	10.24	10.10	10.11	10.35	0.21	0.57	0.40
Isobutyrate	0.40	0.46	0.47	0.49	0.05	0.15	0.52
Valerate	1.05	1.09	1.09	1.12	0.03	0.21	0.75
Isovalerate	0.66[c]	0.72[cd]	0.70[c]	0.80[d]	0.04	0.02	0.57
A:P Ratio	2.56	2.44	2.34	2.34	0.15	0.38	0.57
12 hours							
Total SCFA, *mM*	118.34	124.99	130.41	130.68	6.53	0.05	0.22
Individual SCFA	*mol %*						
Acetate (A)	60.29	61.48	61.06	61.09	1.06	0.41	0.17
Propionate (P)	25.74	25.13	25.42	25.23	1.60	0.27	0.38
Butyrate	11.75	11.36	11.43	11.66	0.69	1.00	0.37
Isobutyrate	0.37	0.38	0.4	0.38	0.04	0.75	0.48
Valerate	1.13	1.12	1.16	1.14	0.04	0.67	0.76
Isovalerate	0.52	0.53	0.53	0.49	0.04	0.29	0.35
A:P Ratio	2.40	2.48	2.44	2.45	0.19	0.33	0.23
24 hours							
Total SCFA, *mM*	156.83	153.0	153.84	157.06	4.87	0.77	0.30
Individual SCFA	*mol %*						
Acetate (A)	55.26	56.17	55.21	55.09	0.50	0.48	0.51
Propionate (P)	26.83[a]	25.29[b]	25.12[b]	24.83[b]	0.89	0.01	0.06
Butyrate	16.05	16.61	17.58	18.03	0.80	0.04	0.57
Isobutyrate	0.34	0.34	0.38	0.37	0.04	0.09	0.50
Valerate	1.01[a]	1.09[ab]	1.15[b]	1.13[b]	0.08	0.01	0.02
Isovalerate	0.51	0.51	0.57	0.54	0.05	0.36	0.48
A:P Ratio	2.06[a]	2.23[b]	2.20[b]	2.24[b]	0.09	0.03	0.09

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.14. Effect of 3 levels (3, 6, and 12 g/kg DM) of sodium sulfate ($NaSO_4$) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	$NaSO_4$, *g/kg DM*					Trend, *p* <	
	0	3	6	12	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	95.61^{cd}	89.82^{c}	98.61^{d}	96.12^{d}	2.56	0.27	0.93
Individual SCFA	*mol %*						
Acetate (A)	62.62	63.27	63.96	63.66	1.46	0.37	0.39
Propionate (P)	23.60	23.24	22.71	22.86	1.14	0.23	0.33
Butyrate	11.41	11.15	10.95	10.99	0.36	0.44	0.58
Isobutyrate	0.50	0.47	0.48	0.49	0.06	0.94	0.63
Valerate	1.20	1.17	1.17	1.20	0.04	0.88	0.51
Isovalerate	0.79	0.81	0.73	0.80	0.08	0.97	0.58
A:P Ratio	2.70	2.76	2.85	2.81	0.21	0.41	0.38
12 hours							
Total SCFA, *mM*	121.80	126.99	121.19	127.32	3.42	0.43	0.79
Individual SCFA	*mol %*						
Acetate (A)	58.47	57.74	56.73	56.84	1.54	0.06	0.23
Propionate (P)	25.52	25.87	26.08	25.85	1.28	0.34	0.14
Butyrate	13.88	14.48	15.16	15.20	0.64	0.17	0.47
Isobutyrate	0.39	0.35	0.38	0.39	0.04	0.52	0.14
Valerate	1.16	1.04	1.10	1.14	0.05	0.79	0.05
Isovalerate	0.58^{a}	0.51^{b}	0.56^{a}	0.58^{a}	0.06	0.13	0.02
A:P Ratio	2.32^{a}	2.25^{ab}	2.20^{b}	2.22^{b}	0.17	0.02	0.03
24 hours							
Total SCFA, *mM*	147.76	159.75	159.78	157.14	3.91	0.22	0.08
Individual SCFA	*mol %*						
Acetate (A)	49.37	50.25	49.01	50.43	1.36	0.53	0.66
Propionate (P)	26.13	26.35	25.76	25.54	1.20	0.17	0.85
Butyrate	22.26	20.93	22.77	21.87	2.23	0.94	0.95
Isobutyrate	0.38^{c}	0.43^{cd}	0.45^{d}	0.39^{c}	0.02	0.86	0.02
Valerate	1.23	1.30	1.27	1.14	0.16	0.14	0.11
Isovalerate	0.62^{c}	0.73^{d}	0.74^{cd}	0.64^{cd}	0.05	0.89	0.01
A:P Ratio	1.90	1.91	1.91	1.98	0.05	0.15	0.54

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.15. Effect of 3 levels (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, $p <$	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.34[a]	0.94[bc]	1.02[ab]	0.62[c]	0.16	0.01	0.72
pH	5.87[d]	5.87[d]	5.85[de]	5.79[e]	0.02	0.02	0.47
NH_3-N, *mg/dL*	8.80	9.31	10.55	11.00	1.25	0.16	0.72
12 hours							
CH_4, *mM*	1.99[d]	1.29[e]	1.38[e]	1.39[e]	0.36	0.09	0.06
pH	5.54[a]	5.43[b]	5.44[b]	5.46[b]	0.02	0.01	0.01
NH_3-N, *mg/dL*	8.22	10.11	9.51	9.32	2.11	0.76	0.52
24 hours							
CH_4, *mM*	1.67	1.05	1.94	1.47	0.44	0.88	0.77
pH	5.21[d]	5.32[e]	5.27[de]	5.28[de]	0.03	0.27	0.11
NH_3-N, *mg/dL*	8.92	9.70	9.64	11.19	1.74	0.06	0.79

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.16. Effect of 3 levels (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, $p <$	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.20[a]	1.21[a]	1.11[a]	0.75[b]	0.21	0.01	0.02
pH	5.82	5.83	5.85	5.85	0.03	0.41	0.74
NH_3-N, *mg/dL*	9.48	12.57	12.72	12.68	2.04	0.34	0.37
12 hours							
CH_4, *mM*	2.29[c]	1.86[cd]	1.52[d]	1.42[d]	0.35	0.02	0.23
pH	5.52[a]	5.40[b]	5.40[b]	5.41[b]	0.03	0.03	0.02
NH_3-N, *mg/dL*	11.18	9.10	8.87	9.67	1.24	0.46	0.21
24 hours							
CH_4, *mM*	1.82	2.06	1.82	1.37	0.55	0.09	0.22
pH	5.21	5.20	5.22	5.23	0.01	0.09	0.71
NH_3-N, *mg/dL*	8.41	9.84	9.78	8.66	1.40	0.96	0.28

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.17. Effect of 3 levels (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, $p <$	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.49[a]	1.12[bc]	1.38[ab]	0.89[c]	0.18	0.01	0.66
pH	5.87	5.87	5.83	5.90	0.02	0.42	0.15
NH_3-N, *mg/dL*	8.13	10.84	10.93	11.36	1.06	0.09	0.21
12 hours							
CH_4, *mM*	1.92	1.66	1.83	1.58	0.32	0.29	0.97
pH	5.50[a]	5.32[b]	5.36[bc]	5.39[c]	0.02	0.05	0.01
NH_3-N, *mg/dL*	7.57	6.34	5.86	8.90	2.08	0.58	0.39
24 hours							
CH_4, *mM*	2.50[a]	1.82[bc]	2.08[b]	1.61[c]	0.43	0.01	0.31
pH	5.12	5.11	5.13	5.13	0.02	0.40	0.76
NH_3-N, *mg/dL*	8.29	3.86	6.07	6.28	1.91	0.78	0.31

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.18. Effect of (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, *p* <	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.07	105.38	97.96	102.49	9.55	0.93	0.86
Individual SCFA	*mol %*						
Acetate (A)	64.27[a]	64.33[a]	63.98[a]	62.38[b]	0.45	0.01	0.22
Propionate (P)	23.98[a]	25.25[a]	25.44[a]	26.79[b]	0.77	0.01	0.71
Butyrate	9.68	8.60	8.77	8.97	0.71	0.51	0.23
Isobutyrate	0.44	0.41	0.38	0.39	0.06	0.61	0.66
Valerate	0.96	0.85	0.86	0.90	0.11	0.78	0.48
Isovalerate	0.68	0.56	0.58	0.58	0.08	0.50	0.45
A:P Ratio	2.69[a]	2.55[a]	2.52[ab]	2.34[b]	0.08	0.01	0.86
12 hours							
Total SCFA, *mM*	128.86	137.19	123.46	135.47	7.16	0.74	0.64
Individual SCFA	*mol %*						
Acetate (A)	61.79	59.14	59.43	59.50	1.02	0.24	0.19
Propionate (P)	26.01	27.89	27.12	27.04	1.26	0.64	0.29
Butyrate	10.23[a]	11.11[ab]	11.58[b]	11.43[b]	0.87	0.02	0.04
Isobutyrate	0.41	0.32	0.30	0.34	0.04	0.29	0.10
Valerate	1.01[a]	1.10[ab]	1.15[ab]	1.24[b]	0.15	0.01	0.57
Isovalerate	0.55	0.43	0.42	0.46	0.05	0.28	0.10
A:P Ratio	2.45	2.13	2.19	2.20	0.14	0.34	0.20
24 hours							
Total SCFA, *mM*	152.92	153.46	154.32	151.59	2.99	0.74	0.60
Individual SCFA	*mol %*						
Acetate (A)	57.95	56.49	57.80	56.53	0.85	0.29	1.00
Propionate (P)	26.06	26.07	25.52	26.38	1.34	0.77	0.48
Butyrate	14.06	15.20	14.54	14.88	0.79	0.63	0.65
Isobutyrate	0.32	0.36	0.35	0.33	0.02	0.93	0.34
Valerate	1.08	1.32	1.26	1.32	0.15	0.14	0.29
Isovalerate	0.52	0.56	0.54	0.55	0.04	0.82	0.72
A:P Ratio	2.23	2.20	2.28	2.17	0.14	0.56	0.41

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.19. Effect of (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, *p* <	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.85	88.63	93.32	84.96	5.77	0.05	0.47
Individual SCFA	*mol %*						
Acetate (A)	62.86	62.90	63.06	62.46	0.80	0.43	0.42
Propionate (P)	24.78	25.30	25.16	25.81	1.29	0.13	0.99
Butyrate	10.24	9.75	9.56	9.72	0.55	0.38	0.30
Isobutyrate	0.40	0.35	0.46	0.35	0.04	0.56	0.34
Valerate	1.05	1.10	1.10	1.06	0.08	0.92	0.41
Isovalerate	0.66[a]	0.60[a]	0.66[a]	0.60[a]	0.03	0.24	0.88
A:P Ratio	2.56	2.50	2.54	2.45	0.16	0.16	0.75
12 hours							
Total SCFA, *mM*	118.33[d]	129.02[e]	128.14[e]	125.19[e]	4.61	0.26	0.03
Individual SCFA	*mol %*						
Acetate (A)	60.48[d]	58.85[e]	59.14[de]	58.32[e]	0.52	0.03	0.33
Propionate (P)	25.74	25.95	26.71	26.82	1.23	0.21	0.68
Butyrate	11.75[a]	13.27[b]	12.28[ac]	12.99[bc]	0.83	0.04	0.21
Isobutyrate	0.37	0.32	0.31	0.28	0.03	0.08	0.61
Valerate	1.13	1.14	1.12	1.18	0.07	0.21	0.31
Isovalerate	0.52[d]	0.47[de]	0.43[e]	0.42[e]	0.02	0.02	0.23
A:P Ratio	2.39	2.29	2.22	2.18	0.13	0.09	0.50
24 hours							
Total SCFA, *mM*	156.83[a]	146.77[ab]	140.45[b]	135.74[b]	4.11	0.0056	0.23
Individual SCFA	*mol %*						
Acetate (A)	55.26	55.01	55.12	54.00	0.82	0.30	0.69
Propionate (P)	26.83[a]	24.95[b]	24.83[b]	25.89[ab]	0.88	0.31	0.01
Butyrate	16.05	17.98	17.97	18.06	1.12	0.18	0.24
Isobutyrate	0.34	0.33	0.33	0.31	0.03	0.40	0.96
Valerate	1.01[d]	1.19[e]	1.21[e]	1.23[e]	0.13	0.03	0.10
Isovalerate	0.51[a]	0.54[a]	0.53[a]	0.51[a]	0.05	0.87	0.63
A:P Ratio	2.06	2.22	2.23	2.09	0.08	0.94	0.03

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 1.20. Effect of (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate (3NPA) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	3NPA, *g/kg DM*					Trend, *p* <	
	0	0.5	1.0	2.0	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.40[a]	93.00[b]	91.80[b]	85.02[c]	3.92	0.01	0.23
Individual SCFA	*mol %*						
Acetate (A)	60.98[a]	64.25[b]	64.26[b]	63.98[b]	0.71	0.02	0.01
Propionate (P)	23.29	23.98	23.89	23.63	1.40	0.82	0.42
Butyrate	12.72[a]	9.89[b]	9.67[b]	10.35[b]	0.79	0.01	0.01
Isobutyrate	0.56	0.34	0.32	0.44	0.09	0.56	0.09
Valerate	1.38[a]	0.95[b]	0.96[b]	0.98[b]	0.11	0.01	0.01
Isovalerate	0.87[a]	0.58[b]	0.60[b]	0.62[b]	0.03	0.01	0.01
A:P Ratio	2.64	2.73	2.71	2.73	0.18	0.54	0.70
12 hours							
Total SCFA, *mM*	121.80	123.41	123.43	116.86	3.32	0.19	0.24
Individual SCFA	*mol %*						
Acetate (A)	58.47	56.56	58.12	58.68	0.65	0.82	0.45
Propionate (P)	25.52	25.33	23.79	25.85	1.09	0.87	0.18
Butyrate	13.88	16.10	16.19	14.59	0.78	0.82	0.02
Isobutyrate	0.39	0.36	0.31	0.33	0.03	0.13	0.20
Valerate	1.16	1.12	1.10	1.07	0.03	0.05	0.68
Isovalerate	0.58	0.53	0.48	0.49	0.03	0.07	0.16
A:P Ratio	2.32	2.23	2.44	2.26	0.12	0.90	0.47
24 hours							
Total SCFA, *mM*	147.76	147.90	145.07	144.06	3.96	0.32	0.95
Individual SCFA	*mol %*						
Acetate (A)	49.37[a]	51.87[b]	53.13[b]	51.67[b]	0.81	0.02	0.01
Propionate (P)	26.13	25.04	24.90	25.17	1.09	0.15	0.06
Butyrate	22.26[d]	21.14[de]	19.96[e]	20.99[d]	1.68	0.11	0.02
Isobutyrate	0.38	0.35	0.33	0.31	0.05	0.27	0.78
Valerate	1.23[de]	1.10[d]	1.12[d]	1.27[e]	0.10	0.22	0.02
Isovalerate	0.62[a]	0.49[b]	0.55[bc]	0.58[ac]	0.02	0.82	0.01
A:P Ratio	1.90[a]	2.08[b]	2.14[b]	2.06[b]	0.07	0.03	0.01

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

1.7 Figures

Figure 1.1. Simplified experimental design of *in-vitro* fermentation per level of sodium nitrate, sodium sulfate, or 3-nitro-1-propionate inclusion in a batch run.

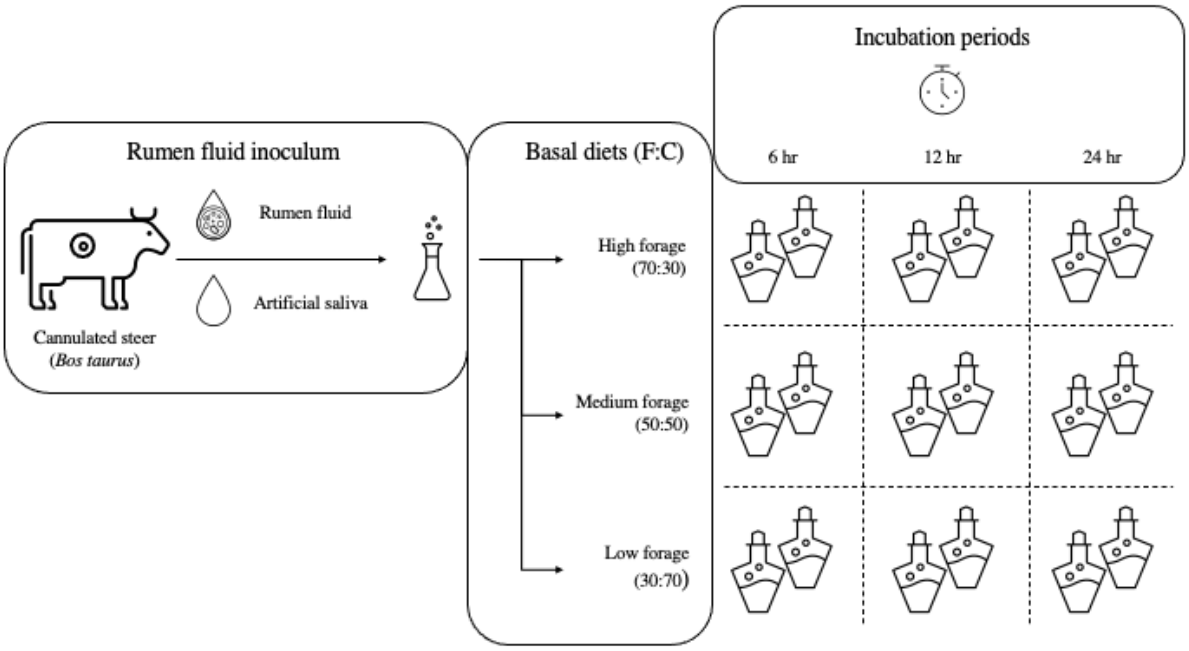
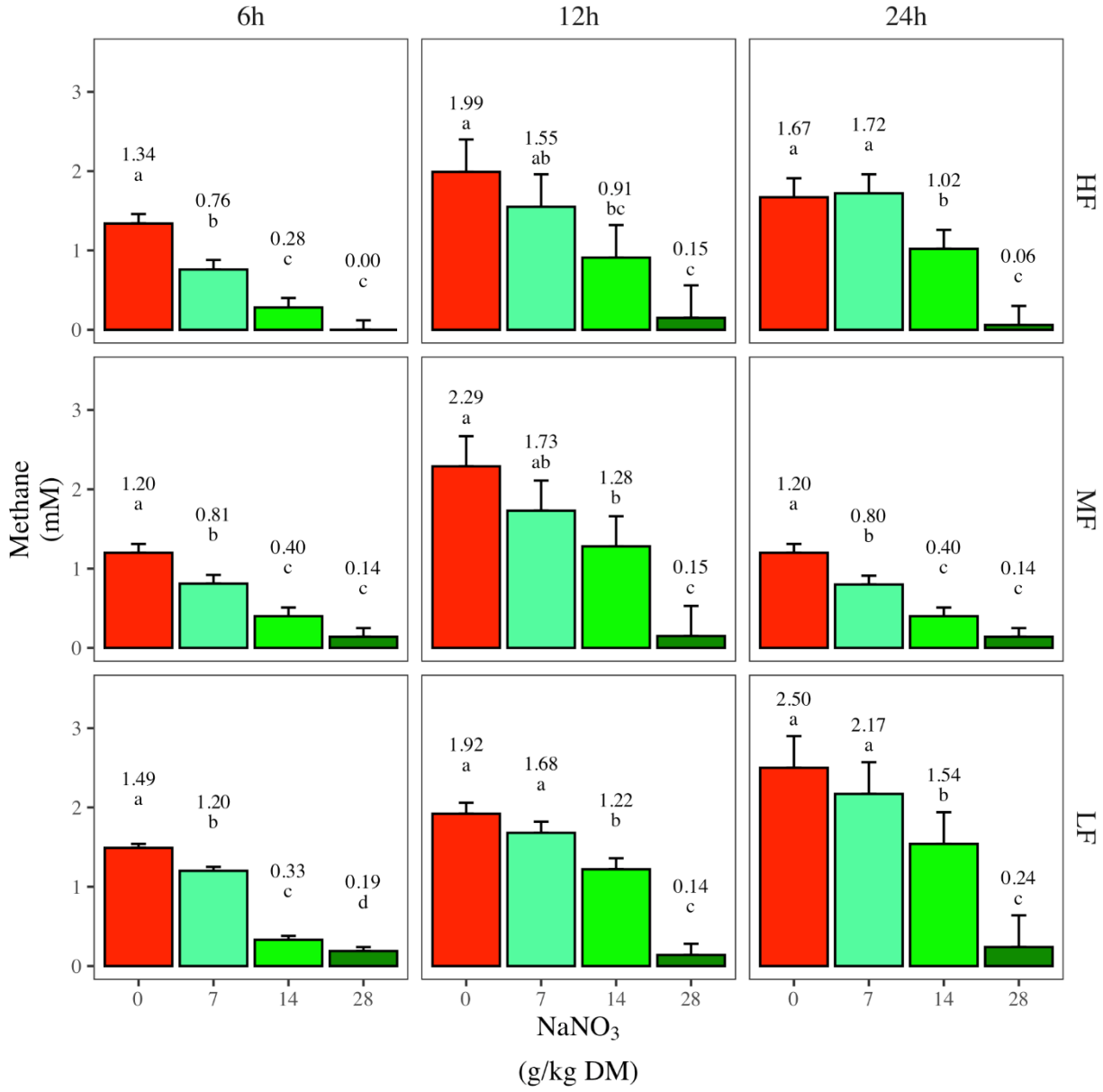
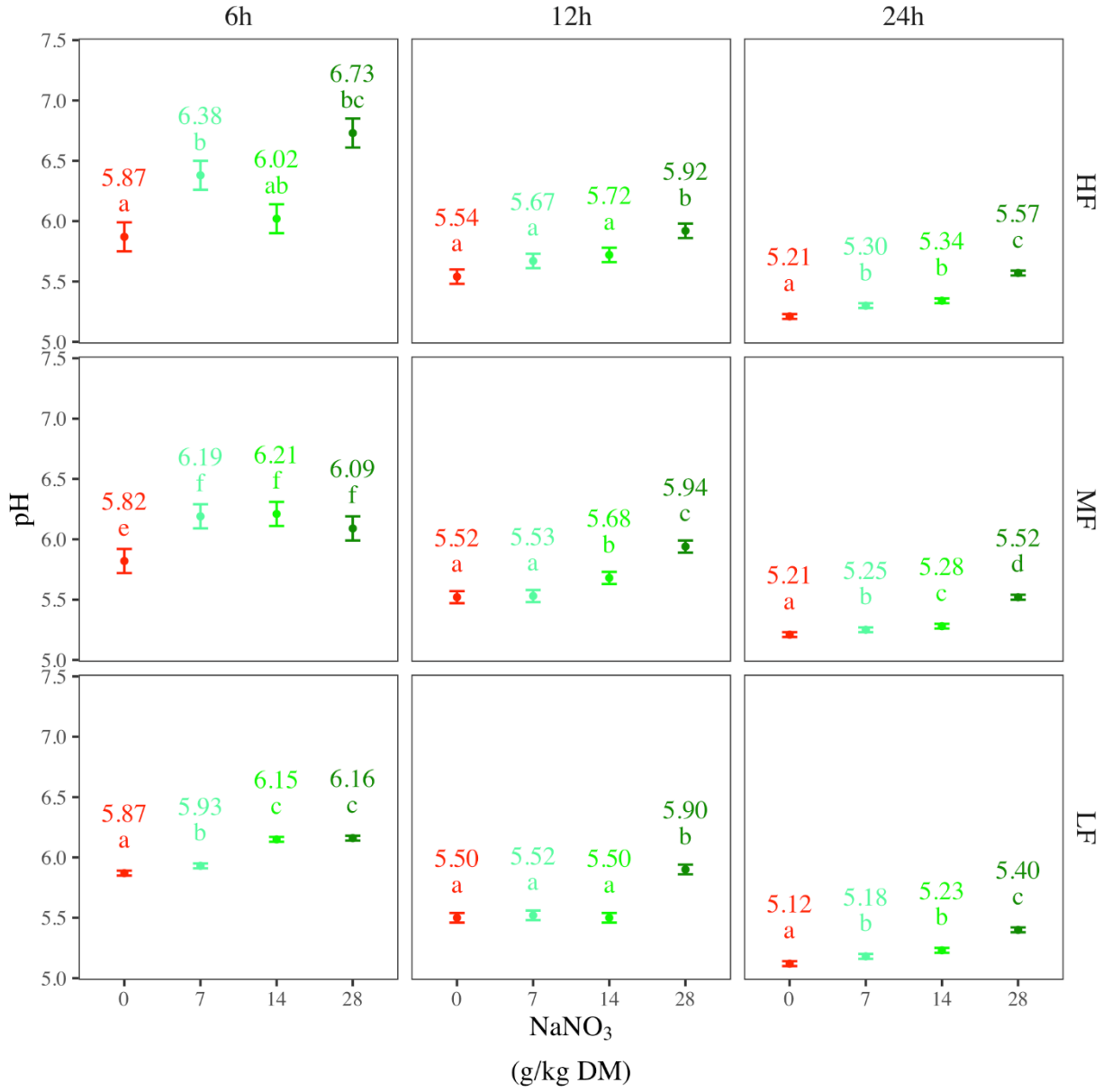


Figure 1.2. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on methane in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



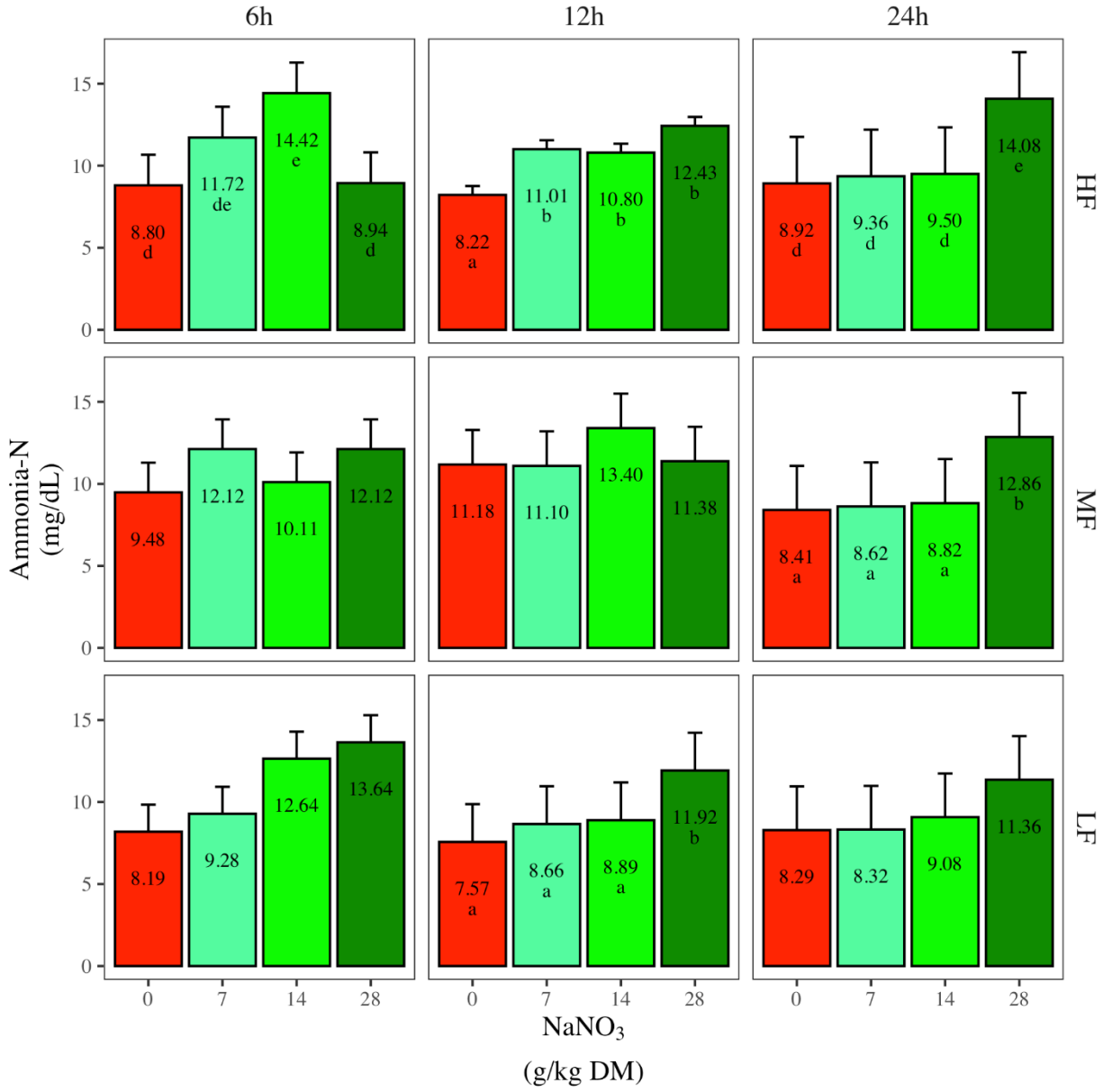
[a-d]different superscripts within a row differ ($p \leq 0.05$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 1.3. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on pH in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a-d]different superscripts within a row differ ($p \leq 0.05$)
[e, f]different superscripts within a row differ ($p \leq 0.10$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 1.4. Effect of 3 levels (7, 14, and 28 g/kg DM) of sodium nitrate ($NaNO_3$) on ammonia-N in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a, b]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 1.5. Simplified schematic representation of the Nap/Nrf complex in *W. succinogenes* (A) and the Nap/Nrf and Nar/Nir complexes in *Selenomonas ruminantium* subsp. *lactilytica* (B).

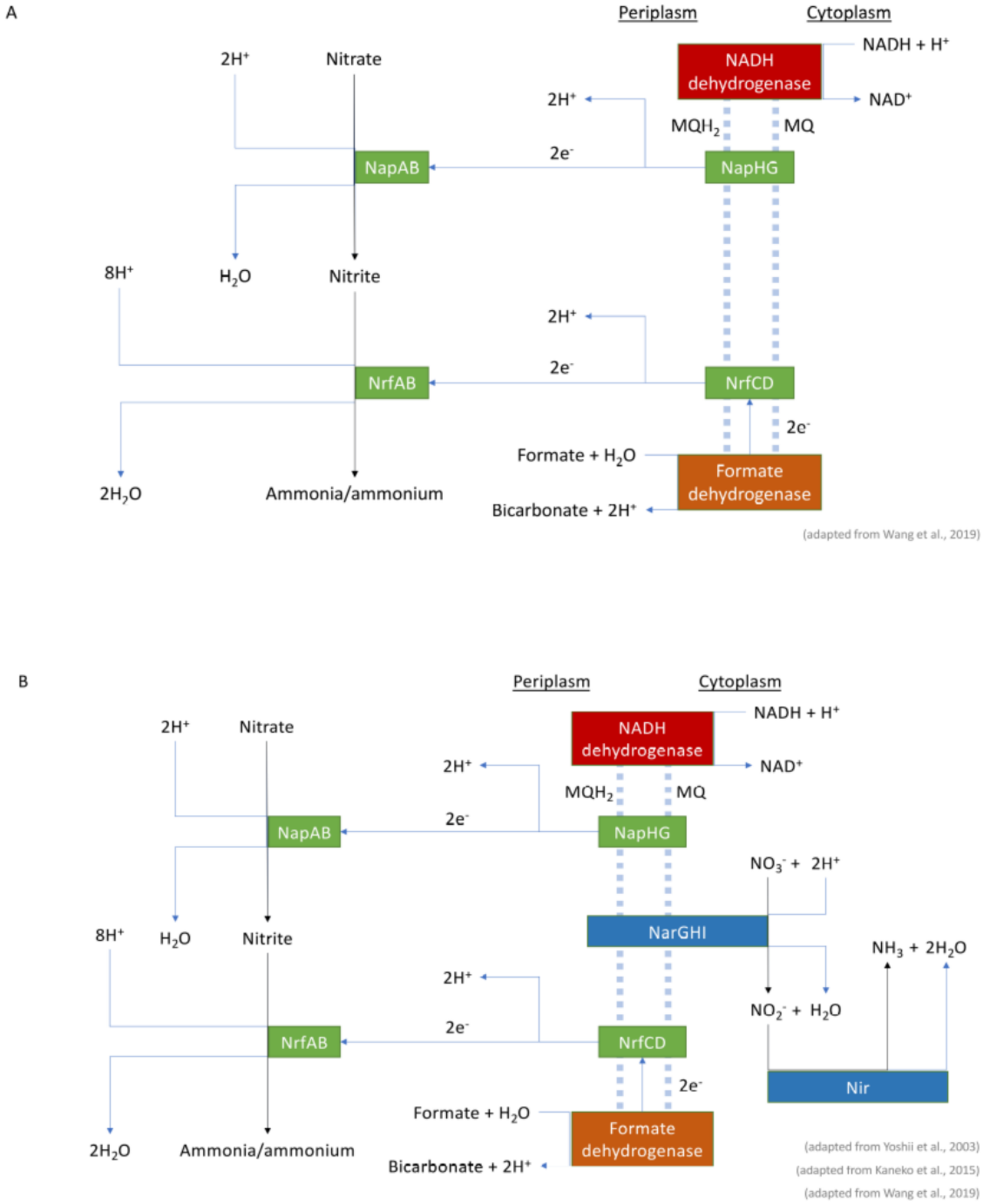


Figure 1.6. Pearson correlation between pH and acetate molar proportion (mol %) at the sodium nitrate ($NaNO_3$) inclusion levels 0, 7, 14, and 28 g/kg DM

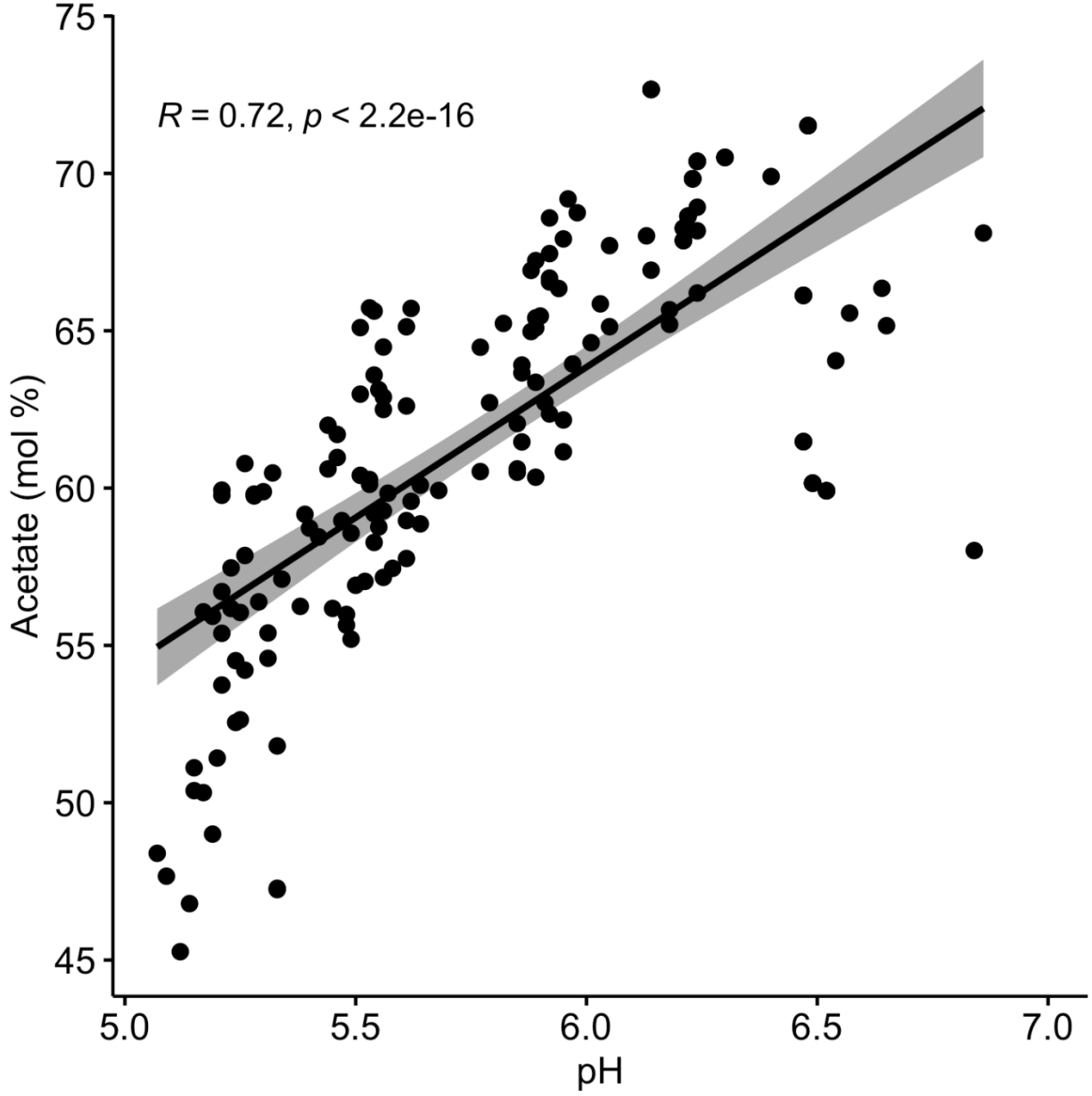


Figure 1.7. Pearson correlation between pH and acetate-to-propionate ratio (A:P) at the sodium nitrate ($NaNO_3$) inclusion levels 0, 7, 14, and 28 g/kg DM

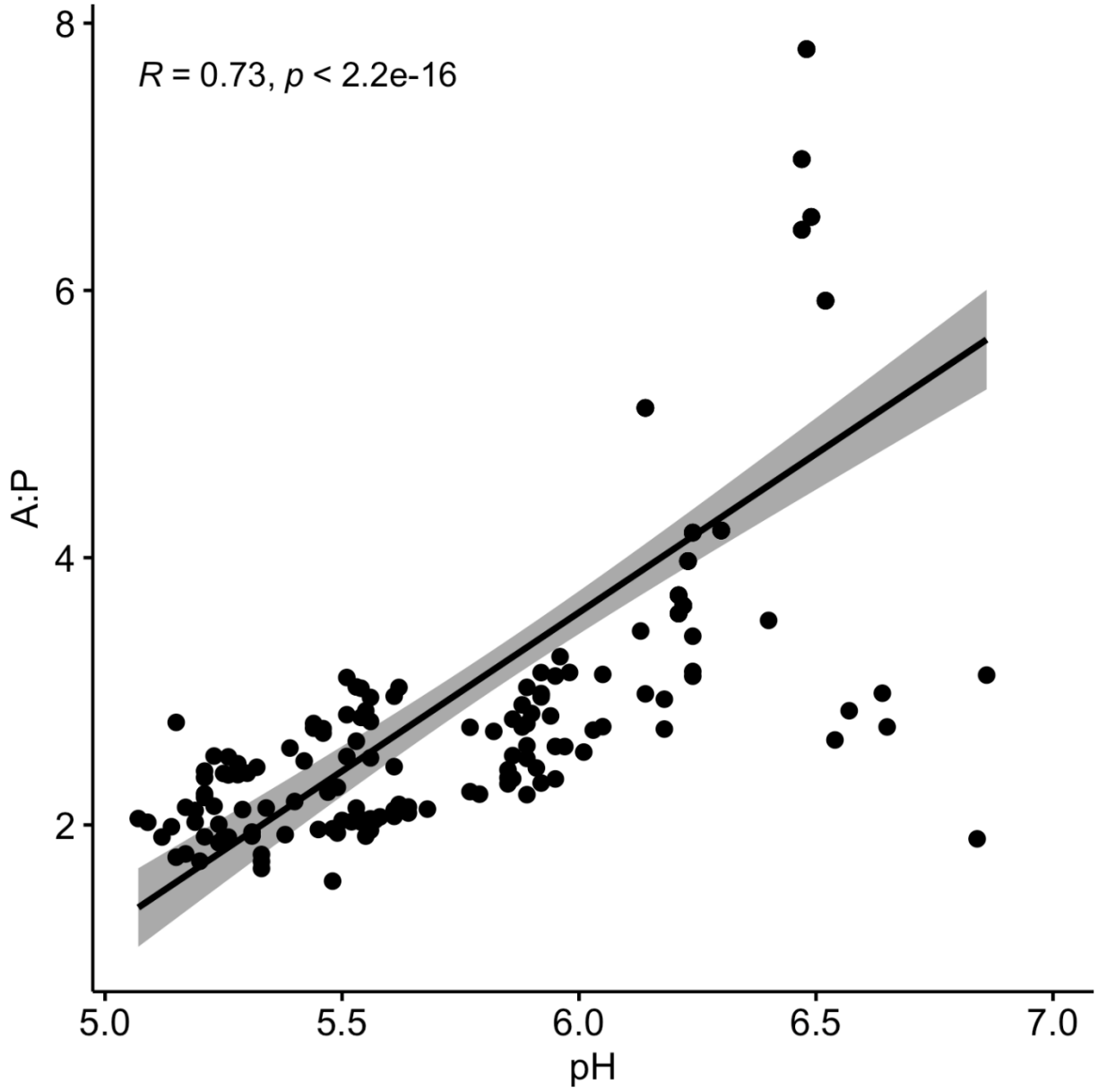
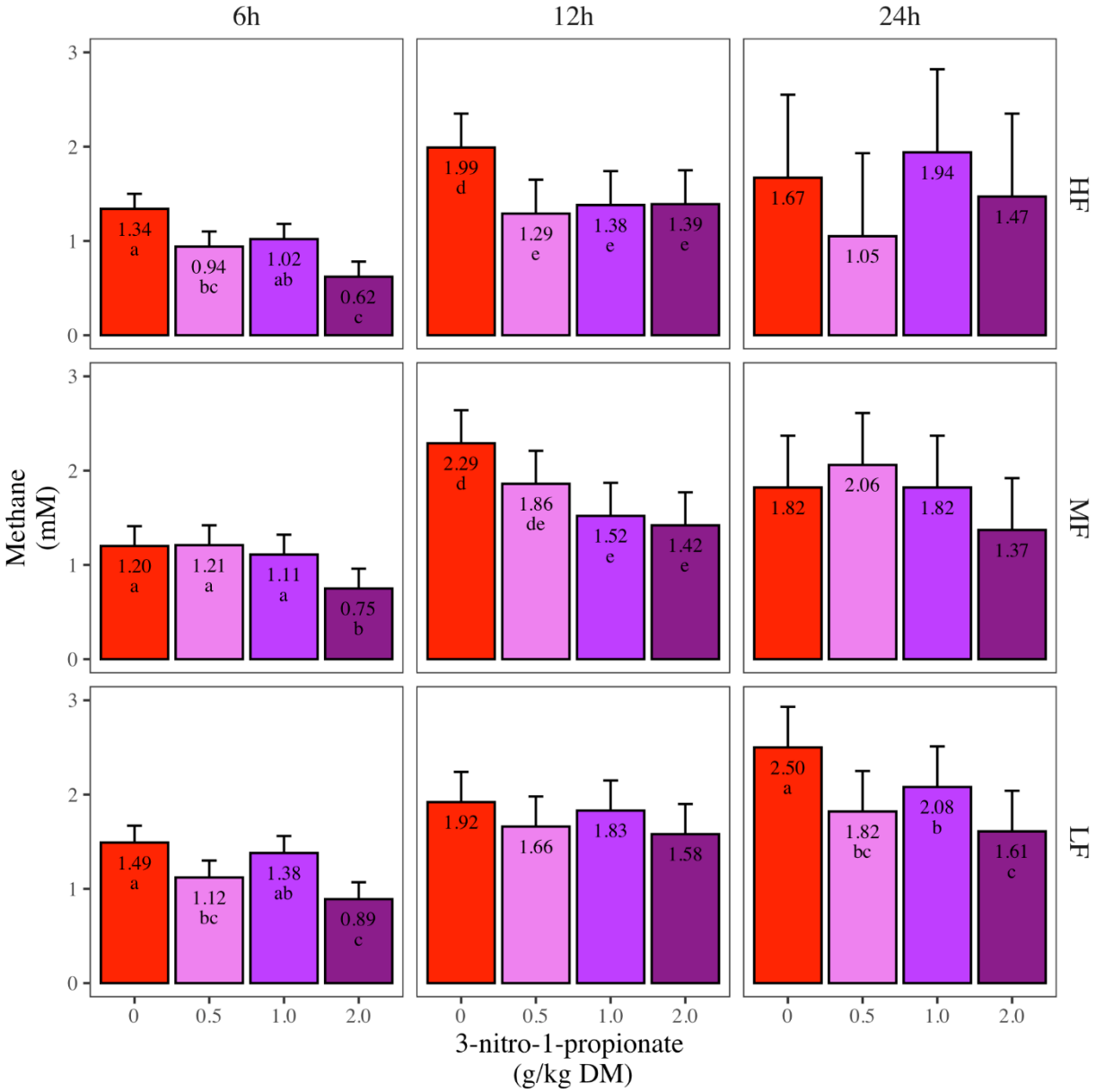
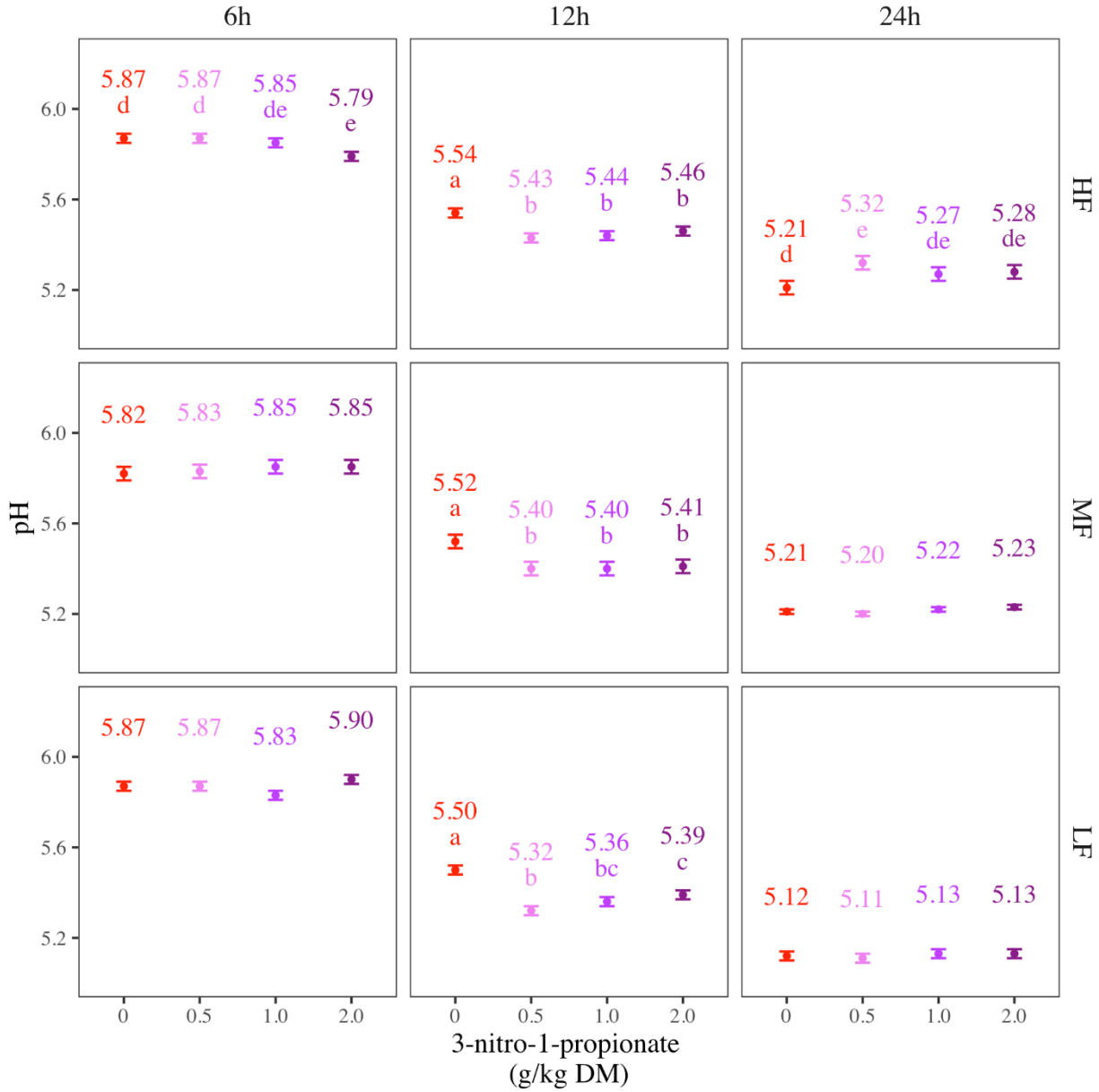


Figure 1.8. Effect of 3 levels (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate on methane in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 1.9. Effect of 3 levels (0.5, 1.0, and 2.0 g/kg DM) of 3-nitro-1-propionate on pH in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 1.10. Pearson correlation between pH and methane (mM) at the 3-nitro-1-propionate inclusion levels 0, 0.5, 1.0, and 2.0 g/kg DM

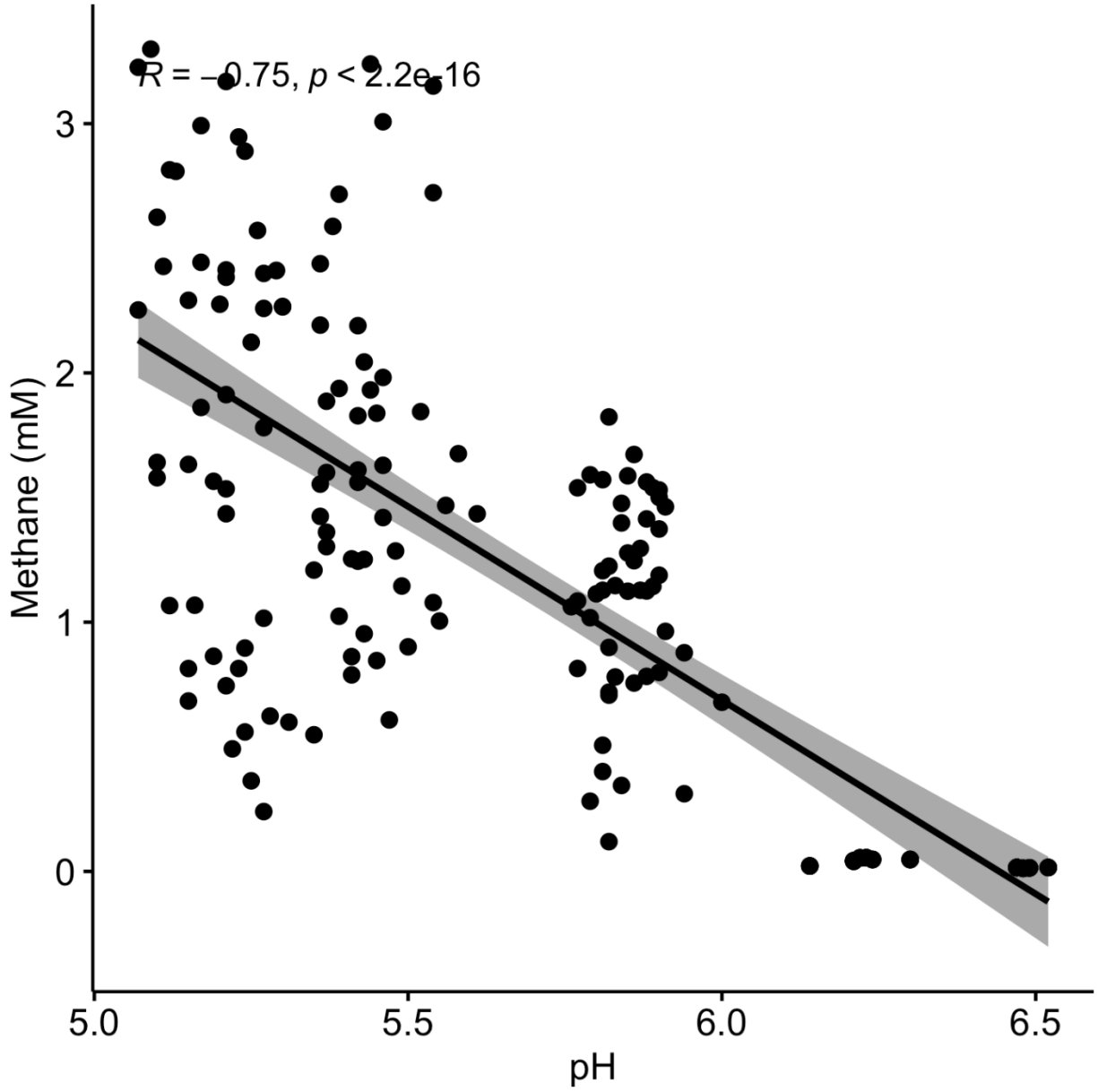
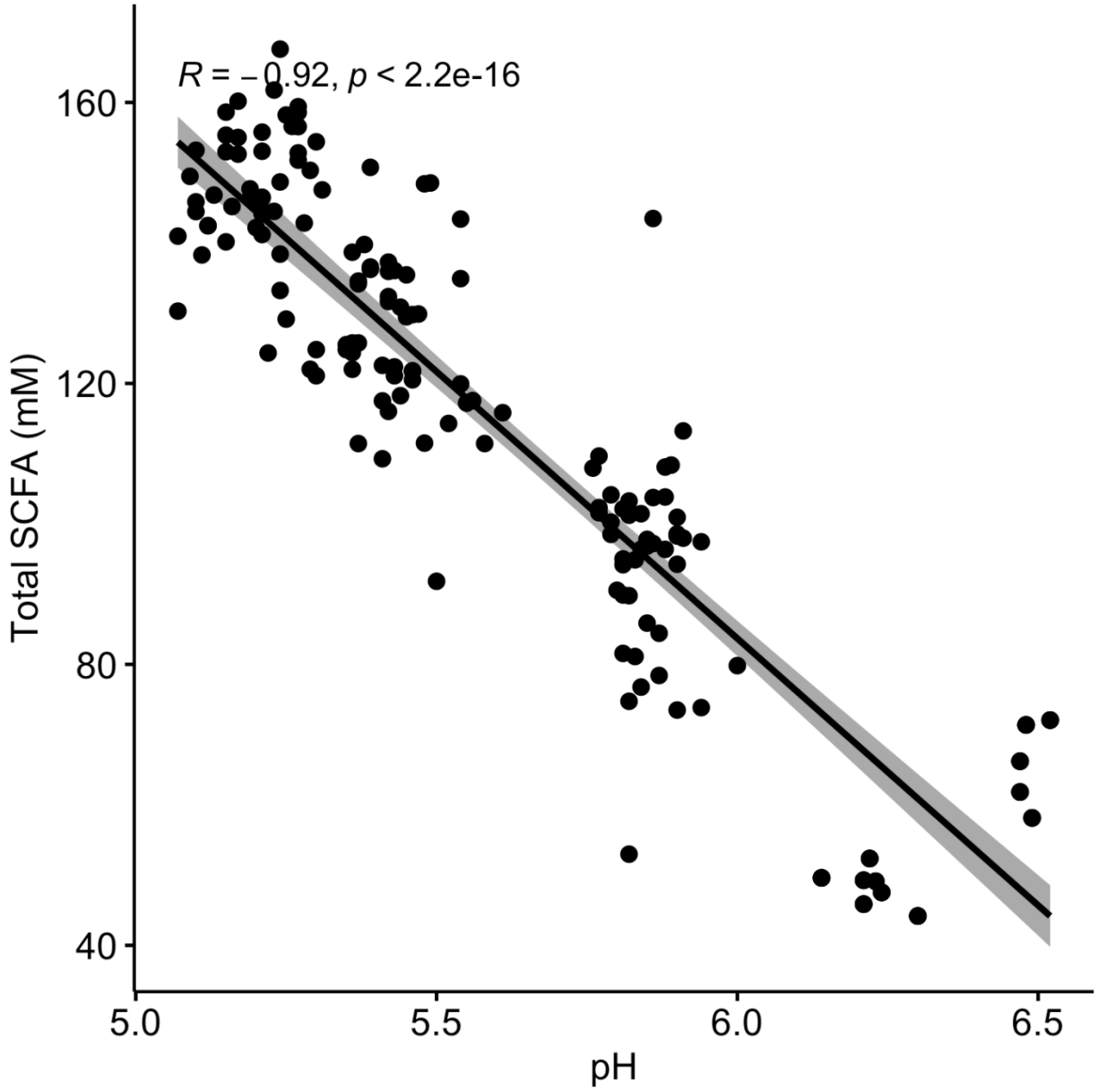


Figure 1.11. Pearson correlation between pH and total short-chain fatty acid concentration (mM) at the 3-nitro-1-propionate inclusion levels 0, 0.5, 1.0, and 2.0 g/kg DM



REFERENCE

Aikman, P. C., Henning, P. H., Humphries, D. J., & Horn, C. H. (2011). Rumen pH and fermentation characteristics in dairy cows supplemented with Megasphaera elsdenii NCIMB 41125 in early lactation. *Journal of dairy science*, *94*(6), 2840–2849. https://doi.org/10.3168/jds.2010-3783

Bach, A., Calsamiglia, S., & Stern, M. D. (2005). Nitrogen Metabolism in the Rumen*. *Journal of Dairy Science*, *88*, E9–E21. https://doi.org/10.3168/jds.S0022-0302(05)73133-7

Beecher, G. R., & Whitten, B. K. (1970). Ammonia determination: Reagent modification and interfering compounds. *Analytical Biochemistry*, *36*(1), 243–246. https://doi.org/10.1016/0003-2697(70)90356-8

Blaxter, K., & Clapperton, J. (1965). Prediction of the amount of methane produced by ruminants. *British Journal of Nutrition, 19*(1), 511-522. doi:10.1079/BJN19650046

Chen, G., & Russell, J. B. (1989). More monensin-sensitive, ammonia-producing bacteria from the rumen. *Applied and environmental microbiology*, *55*(5), 1052–1057. https://doi.org/10.1128/aem.55.5.1052-1057.1989

Denger, K., & Schink, B. (1992). Energy conservation by succinate decarboxylation in Veillonella parvula. *Journal of general microbiology, 138*(5), 967-71.

De Raphélis-Soissan, V., Li, L., Godwin, I.R., Barnett, M., Perdok, H., & Hegarty, R. (2014). Use of nitrate and Propionibacterium acidipropionici to reduce methane emissions and increase wool growth of Merino sheep. *Animal Production Science*, *54*(10), 1860-1866. 10.1071/AN14329.

EPA (2022) Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2020. U.S. Environmental Protection Agency, EPA 430-R-22-003.

https://www.epa.gov/ghgemissions/draft-inventory-us-greenhouse-gas-emissionsand-sinks-1990-2020.

Farra, P. A., and Satter, L. D. (1971). Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *Journal of Dairy Science*, *54*, 1018–1024. doi: 10.3168/jds.S0022-0302(71) 85965-9

Gupta, V.P., Kamra, D.N., Agarwal, N., & Chaudary, L. (2017). Effect of sulphate and blend of plant parts containing secondary metabolites on in vitro methanogenesis, digestibility and feed fermentation with buffalo rumen liquor. *87*. 199-202.

Hackmann, T. J., Ngugi, D. K., Firkins, J. L., & Tao, J. (2017). Genomes of rumen bacteria encode atypical pathways for fermenting hexoses to short-chain fatty acids. *Environmental Microbiology*, *19*(11), 4670–4683. https://doi.org/10.1111/1462-2920.13929

Hino, T., & Kuroda, S. (1993). Presence of lactate dehydrogenase and lactate racemase in Megasphaera elsdenii grown on glucose or lactate. *Applied and environmental microbiology*, *59*(1), 255–259. https://doi.org/10.1128/aem.59.1.255-259.1993

Hulshof, R. B., Berndt, A., Gerrits, W. J., Dijkstra, J., van Zijderveld, S. M., Newbold, J. R., & Perdok, H. B. (2012). Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. *Journal of animal science*, *90*(7), 2317–2323. https://doi.org/10.2527/jas.2011-4209

Iwamoto, M., Asanuma, N., & Hino, T. (2002). Ability of Selenomonas ruminantium, Veillonella parvula, and Wolinella succinogenes to Reduce Nitrate and Nitrite with Special Reference to the Suppression of Ruminal Methanogenesis. *Anaerobe*, *8*(4), 209–215. https://doi.org/10.1006/anae.2002.0428

Janssen, P.H. Growth yield increase and ATP formation linked to succinate decarboxylation in *Veillonella parvula*. *Arch. Microbiol.* **157,** 442–445 (1992). https://doi.org/10.1007/BF00249102

Johns, A.T. (1951). The mechanism of propionic acid formation by Veillonella gazogenes. *Journal of general microbiology, 5*(2), 326-36

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science*, *73*(8), 2483–2492. https://doi.org/10.2527/1995.7382483x

Kandylis, K. (1984). The role of sulphur in ruminant nutrition. A review. *Livestock Production Science*, *11*. 611-624.

Kanegasaki, S., & Takahashi, H. (1967). Function of growth factors for rumen microorganisms. I. Nutritional characteristics of Selenomonas ruminantium. *Journal of bacteriology*, *93*(1), 456–463. https://doi.org/10.1128/jb.93.1.456-463.1967

Kaneko, J., Yamada-Narita, S., Abe, N., Onodera, T., Kan, E., Kojima, S., Miyazaki, T., Yamamoto, Y., Oguchi, A., Ankai, A., Ichikawa, N., Nakazawa, H., Fukui, S., Takahashi, M., Yamazaki, S., Fujita, N., & Kamio, Y. (2015). Complete genome sequence of Selenomonas ruminantium subsp. Lactylitica will accelerate further understanding of the nature of the class Negativicutes. *FEMS Microbiology Letters*, *362*(9), fnv050. https://doi.org/10.1093/femsle/fnv050

Kassambra, A. (2020). ggpubr: *'ggplot2' Based Publication Ready Plots. R package version 0.4.0, <https://CRAN.R-project.org/package=ggpubr>*.

Kohn, R. A., & Dunlap, T. F. (1998). Calculation of the buffering capacity of bicarbonate in the rumen and in vitro. *Journal of Animal Science*, *76*(6), 1702–1709. https://doi.org/10.2527/1998.7661702x

Kröger, A., Biel, S., Simon, J., Gross, R., Unden, G., & Lancaster, C. R. D. (2002). Fumarate respiration of Wolinella succinogenes: Enzymology, energetics and coupling mechanism. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1553*(1), 23–38. https://doi.org/10.1016/S0005-2728(01)00234-1

Latham, E. A., Anderson, R. C., Pinchak, W. E., & Nisbet, D. J. (2016). Insights on Alterations to the Rumen Ecosystem by Nitrate and Nitrocompounds. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00228

Lee, C., Araujo, R. C., Koenig, K. M., & Beauchemin, K. A. (2017). In situ and in vitro evaluations of a slow-release form of nitrate for ruminants: Nitrate release rate, rumen nitrate metabolism and the production of methane, hydrogen, and nitrous oxide. *Animal Feed Science and Technology*, *231*, 97–106. https://doi.org/10.1016/j.anifeedsci.2017.07.005

Majak, W., & Cheng, K. J. (1981). Identification of rumen bacteria that anaerobically degrade aliphatic nitrotoxins. *Canadian journal of microbiology*, *27*(7), 646–650. https://doi.org/10.1139/m81-099

McDougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *The Biochemical Journal*, *43*(1), 99–109.

Melville, S. B., Michel, T. A., & Macy, J. M. (1988). Pathway and sites for energy conservation in the metabolism of glucose by Selenomonas ruminantium. *Journal of bacteriology*, *170*(11), 5298–5304. https://doi.org/10.1128/jb.170.11.5298-5304.1988

Moe, P. W., & Tyrrell, H. F. (1979). Methane Production in Dairy Cows. *Journal of Dairy Science*, *62*(10), 1583–1586. https://doi.org/10.3168/jds.S0022-0302(79)83465-7

National Research Council. (2001). *Nutrient Requirements of Dairy Cattle: Seventh Revised Edition, 2001*. Washington, DC: The National Academies Press. https://doi.org/10.17226/9825.

Odle, J., & Schaefer, D. (1987). Influence of rumen ammonia concentration on the rumen degradation rates of barley and maize. *British Journal of Nutrition, 57*(1), 127-138. doi:10.1079/BJN19870016

Ohashi, Y., Ushida, K., Miyasaki, K., & Kojima, Y. (1996). Effect of initial sulfate level on electron partition between methanogenesis and sulfate reduction in the rumen. *Annales de Zootechnie*, *45*(Suppl. 1), 320–320. https://doi.org/10.1051/animres:19960660

Olijhoek, D. W., Hellwing, A., Brask, M., Weisbjerg, M. R., Højberg, O., Larsen, M. K., Dijkstra, J., Erlandsen, E. J., & Lund, P. (2016). Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *Journal of dairy science*, *99*(8), 6191–6205. https://doi.org/10.3168/jds.2015-10691

Paynter, M. J., & Elsden, S. R. (1970). Mechanism of propionate formation by Selenomonas ruminantium, a rumen micro-organism. *Journal of general microbiology*, *61*(1), 1–7. https://doi.org/10.1099/00221287-61-1-1

Rimington, C., & Quin, J.I. (1933). Studies on the photosensitisation of animals in South Africa. II. The presence of a lethal factor in certain members of the plant genus Tribulus. *The Onderstepoort journal of veterinary science and animal industry, 1*, 469-489.

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

Stewart, C.S., Flint, H.J., Bryant, M.P. (1997). The rumen bacteria. In: Hobson, P.N., Stewart, C.S. (eds) The Rumen Microbial Ecosystem, 10-72. https://doi.org/10.1007/978-94-009-1453-7_2

Thauer, R. K., Jungermann, K., & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological reviews*, *41*(1), 100–180. https://doi.org/10.1128/br.41.1.100-180.1977

Ungerfeld, E., & Kohn, R. (2006). The Role of Thermodynamics in the Control of Ruminal Fermentation. In *Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*. 55–85.

Van Lingen, H. J., Fadel, J. G., Yáñez-Ruiz, D. R., Kindermann, M., & Kebreab, E. (2021). Inhibited Methanogenesis in the Rumen of Cattle: Microbial Metabolism in Response to Supplemental 3-Nitrooxypropanol and Nitrate. *Frontiers in microbiology*, *12*, 705613. https://doi.org/10.3389/fmicb.2021.705613

Van Nevel, C. J., & Demeyer, D. I. (1996). Control of rumen methanogenesis. *Environmental Monitoring and Assessment, 42*(1), 73–97. https://doi.org/10.1007/BF00394043

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

Veneman, J. B., Muetzel, S., Hart, K. J., Faulkner, C. L., Moorby, J. M., Perdok, H. B., & Newbold, C. J. (2015). Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and Animal Productivity in Dairy Cows?. *PloS one*, *10*(10), e0140282. https://doi.org/10.1371/journal.pone.0140282

Wang, X., Tamiev, D., Alagurajan, J., Dispirito, A., Phillips, G., & Hargrove, M. (2019). The role of the NADH-dependent nitrite reductase, Nir, from Escherichia coli in fermentative ammonification. *Archives of Microbiology*, *201*. https://doi.org/10.1007/s00203-018-1590-3

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Williams, M. C., James, L. F., & Bleak, A. T. (1976). Toxicity of introduced nitro-containing Astragalus to sheep, cattle, and chicks. *Journal of Range Management*, *29*(1), 30-33.

Yoshii, T., Asanuma, N., & Hino, T. (2003). Number of nitrate- and nitrite-reducing Selenomonas ruminantium in the rumen, and possible factors affecting its growth. *Animal Science Journal*, *74*(6), 483–491. https://doi.org/10.1046/j.1344-3941.2003.00142.x

Zhao, Y., Xie, B., Gao, J., & Zhao, G. (2020). Dietary supplementation with sodium sulfate improves rumen fermentation, fiber digestibility, and the plasma metabolome through modulation of rumen bacterial communities in steers. *Applied and environmental microbiology*, *86*(22), e01412-20.

CHAPTER 2: Effect of bromochloromethane on *in-vitro* ruminal methanogenesis and fermentation pattern

2.1 Abstract

There has been an increasing amount of research efforts on mitigation of enteric methane (CH_4) emission as CH_4 is considered a disservice both nutritionally and environmentally. CH_4 represents up to 10% of energy that would otherwise benefit animal growth and performance. CH_4 is a greenhouse gas and contributes to anthropogenic carbon footprint of the livestock industry. The use of halogenated CH_4 analogues such as bromochloromethane (BCM) is one of the most effective methods of CH_4 inhibition. Here, we investigated the effect of BCM on *in-vitro* methanogenesis and fermentation pattern by varying inclusion levels and basal diets.

The experimental design included 4 levels of BCM (0, 0.075, 0.15, and 0.30 g/kg DM) on high (HF, 70:30 forage to concentrate, F:C), medium (MF, 50:50 F:C), and low (LF, 30:70 F:C) forage diets. 100-mL glass bottles with septum caps contained each combination of BCM and diet and were inoculated with 30 mL of rumen fluid from a cannulated Hereford steer. After 6, 12, and 24 h of incubation at 39°C, *in-vitro* fermentation was terminated in ice and the contents of the bottles were analyzed for CH_4, pH, NH_3-N, and SCFAs.

Inclusion of BCM achieved between 95 and 99% inhibition of CH_4 in all levels. pH decreased at 12 h compared to the control but increased or did not differ from the control at 24 h. NH_3-N was largely unaffected by BCM. Total SCFA decreased numerically, significantly for the HF and MF diets, respectively, and tended to decrease for the LF diet. Acetate to propionate ratio significantly decreased for all diets. The result of the present study demonstrates the potent anti-methanogenic effect of direct enzymatic inhibition of methanogenesis.

2.2 Introduction

From the standpoint of human food consumption, the ruminant converts inedible plant matter into nutritious animal products such as meat and milk. Therefore, the ruminant serves as the nexus between the lower and upper trophic levels in the ecosystem. This conversion of the inedible to the edible nutrients primarily occurs in the rumen. The mammalian digestive physiology cannot process plant structural carbohydrates such as cellulose and hemicellulose, but ruminal microorganisms conduct chains of metabolic reactions intra- and intercellularly to reduce these polymers to simple organic molecules. In turn, the products of microbial fermentation in the rumen namely short-chain fatty acids are the main source of energy for the ruminant host, establishing a commensal symbiosis.

Methane (CH_4) in the rumen is considered a means of hydrogen (H_2) disposal and thus maintains the ruminal fermentation by preventing the accumulation of H_2 (Johnson & Johnson, 1995). However, from the nutritional perspective, this functional role of CH_4 in the rumen also represents an 2-10% energy loss via eructation (Blaxter & Clapperton, 1965). Additionally, enteric emission of CH_4 from ruminant livestock poses an environmental concern and contributes to 26.9% of all anthropogenic CH_4 emissions in the US (EPA, 2020). First as an effort to improve feed efficiency and later to alleviate the environmental impacts of CH_4, multiple strategies have been considered to reduce or terminate CH_4, of which the most effective is the use of halogenated methane analogues such as bromochloromethane (BCM; Chalupa, 1977; Sirohi & Goel, 2013; Matsui et al., 2020).

The use of BCM for its anti-methanogenic property has been studied *in vitro* (Trei et al., 1970) and *in vivo* (Sawyer et al., 1974; McCrabb et al., 1997; Tomkins & Hunter, 2004 Tomkins et al., 2009; Abecia et al., 2012) both before and after its encapsulation with α-cyclodextrin (CD)

was patented by May et al. (1996). While the BCM-CD formulation allows for sustained effect and prevention of its ozone depleting effect (Abecia et al., 2012), the anti-methanogenic efficacy of BCM, regardless of encapsulation, stems from its mode of action specifically targeting the reduced form of vitamin B_{12} and thus inhibiting the cobamide-dependent methyl transferase (Wood et al., 1968). However, the effect of energy levels from the basal diet on the anti-methanogenic effects of BCM has not been investigated.

Therefore, the present study examined the effect of three different levels of BCM on *in-vitro* methanogenesis and fermentation parameters in mixed cultures of rumen microorganisms. The inclusion of BCM was conducted on three basal diets differing in forage-to-concentrate ratio (F:C) and thus energy levels, which has long been known to alter fermentation pattern (Blaxter and Clapperton, 1965; Johnson & Johnson, 1997).

2.3 Material and Methods

Feed Additives and Feed Substrates

To test the anti-methanogenic efficacy of BCM, experiments were designed to include four different levels of BCM and three different diets in *in-vitro* batch cultures of mixed rumen microbes. The four levels included a control (0), 0.075, 0.15, & 0.30 g/kg DM BCM. The three diets were formulated with alfalfa hay and concentrate mix for the following F:C: 70:30 for high forage (HF); 50:50 for medium forage (MF); and 30:70 for low forage (LF). The concentrate mix consisted of a mixture of ground corn, soybean meal, and vitamin and mineral mix. The ingredients and chemical composition of the basal diets are summarized in tables 2.1 and 2.2, respectively. Each experimental diet (approx. 1.0 g) was quantitatively weighed in a 100-mL glass culture bottle, in duplicates, and incubated for 6, 12, and 24 h at 39°C. The experiment was replicated twice (n=2) for statistical analysis (figure 2.1).

Rumen Fluid Collection and Inoculation

Rumen inoculum was prepared by mixing rumen fluid and artificial saliva in a 1:2 ratio. The rumen fluid was collected from a cannulated Hereford steer (*Bos taurus*), whose basal diet was a total mixed ration on a perennial grass pasture at the NCSU Metabolic Unit. The artificial saliva was prepared according to the ruminant salivary composition by McDougall (1948) and consisted of $NaHCO_3$, $NaH_2PO_4 \bullet H_2O$, NaCl, KCl, $CaCl \bullet 2H_2O$, $MgCl_2 \bullet 6H_2O$, and Urea. Thirty mL of rumen inoculum were allocated to each fermentation bottle containing 1.0 g of feed substrate and each level of a feed additive while the bottle was flushed with a stream of CO_2 prior to and during inoculation to maintain anaerobicity. Immediately after the inoculation, the bottles were sealed with rubber-lined septum caps and placed in a water bath at 39ºC for 6, 12, and 24 h. After each time period, respective bottles were transferred to ice bath to terminate the *in-vitro* microbial activity.

Analytical Measurements

The present study measured CH_4, pH, ammonia-N (NH_3-N), and short-chain fatty acids (SCFAs) at 6, 12, and 24 h of *in-vitro* fermentation. Methane concentration in the fermentation bottle was measured using a gas chromatography (model CP-3800; Varian, Walnut Creek, CA) using a stainless-steel column packed with Molsieve 5A 45/60 mesh (Supelco Inc., Bellefonte, PA). Gas samples were withdrawn from the headspace (70-mL) within each bottle with an air-tight syringe (Hamilton Co., Reno, NV). The rubber-lined septum cap retained the gas within the headspace while taking samples.

After CH_4 measurement, pH of the culture fluid in the bottle was measured with a pH probe (VWR SympHony – model AR25; Accumet Research, Dual Channel pH/Ion Meter Fisher Scientific). Following pH measurements, culture contents were transferred to a tube and

centrifuged at 2,000 rpm for 5 minutes to separate the solid digesta from the liquid. Four mL of supernatant were aliquoted to a 5.0 mL centrifuge tube and was kept in a freezer at - 20ºC for subsequent NH_3-N and SCFA analyses. After thawing the 4.0-mL of rumen fluid, two 1.0-mL aliquots of culture fluids were transferred into separate microcentrifuge tubes for NH_3-N and SCFA analyses.

NH_3-N concentration was calculated Using the colorimetric procedure outlined by Beecher and Whitten (1970). Standards containing 0, 4, 8, 12, and 16 μg/mL of NH_3-N were prepared. Samples were centrifuged at 15,000 rpm for 15 minutes to separate any remaining solid particles from the liquid. For the analysis, 5.0 μL of each sample or standards were transferred in duplicates into glass tubes. One hundred μL of DI water, and 0.5 mL of phenol and sodium hypochlorite reagents were added. The samples and standards were allowed to react for 30 minutes at room temperature. Following 30 min, 4.0 mL of DI water were added to the sample mixture. The sample was then transferred to a cuvette for absorbance measured at wavelength of 630 nm. The standards were used to determine the concentration of unknown samples.

Concentration of SCFA was measured by gas chromatography (model CP- 3380; Varian, Walnut Creek, CA) using a fused silica capillary column (Nukol™; Superlco Inc., Bellefonte, PA). One mL aliquots of culture contents were frozen, thawed, and centrifuged at 15,000 rpm for 15 minutes to separate remaining solid particle from the liquid. The 1.0-mL sample aliquot was treated with 0.2 mL of a metaphosphoric acid, which included 2-ethylbutyrate as internal standard. The sample was then centrifuged at 15,000 rpm for 5 minutes and transferred to a GC vial. The column used in this study detected lactate, acetate, propionate, butyrate, valerate, and isoacids the (isobutyrate and isovalerate).

Calculation of Hydrogen Recovery

The rate of hydrogen recovery was calculated according to Van Soest's (1994) modification of Wolin's formula (1960) as follows:

$$H_2\ recovery\ (\%) = \left(\frac{4 \times CH_4 + 2 \times Propionate + 2 \times Butyrate + Valerate}{2 \times Acetate + Propionate + 4 \times Butyrate + 2 \times Isobutyrate + 2 \times Valerate}\right) \times 100$$

The values of CH_4 and individual SCFAs used in the equation are converted to mmol by multiplying their concentrations with the rumen liquid volume of 30 mL and headspace volume of 70 mL in the bottle for CH_4 and individual SCFAs, respectively.

Statistical Analysis

Data from above measurements was analyzed according to a completely randomized block design using the Mixed procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Data from 6, 12, and 24 h were analyzed separately. The model included the main effect of additive levels. Orthogonal contrasts were used to determine linear or quadratic trends. The replicate variable nested within the batch run was treated as a random effect. Thus, the model is represented by:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(k)} + \varepsilon_{ijk}$$

where

y_{ijk} = each response variable measured,

μ = overall mean,

α_i = fixed effect of level

$\beta_{j(k)}$ = random effect of replicate nested within run

ε_{ijk} = subplot error.

Significant effects were declared at *p-value* ≤ 0.05 and tendencies at ≤ 0.10. The analyzed data were visualized in figures using the following R packages: ggplot2 (Wickham, 2016) and ggpubr (Kassambra, 2020).

2.4 Results

Effect of BCM on CH_4, pH, and NH_3-N

The data for the effect of BCM on CH_4, pH, and NH_3-N are summarized in tables 2.3-2.5. At 6 h, CH_4 significantly decreased by 95, 97, and 98% of the control value in the 0.075, 0.15, and 0.30 g/kg DM BCM inclusion levels, respectively, regardless of the diet (table 2.3-2.5). Even at the lowest inclusion level, BCM had a drastic inhibition on methanogenesis (table 2.3-2.5). At 12 and 24 h, CH_4 was mitigated by 98-99% of the control values in the BCM treatment while its level effect was quadratic (tables 2.3-2.5).

At 6 h, pH was not affected by the BCM treatment at 6 h across all diets (tables 2.3-2.5). However, by 24 h, pH increased in the HF diet and tended to remain relatively unchanged in the MF diet; in the LF diet BCM had a quadratic effect with an increase at the low level of inclusion and then a decrease at the high level (tables 2.3-2.5).

There was no major effect of BCM inclusion levels on NH_3-N, except for a significant linear increase at 6 h of incubation with the LF diet (table 2.5).

Effect of BCM on H_2 Recovery

The data for the effect of BCM on H_2 recovery rate are summarized in table 2.6. BCM treatment decreased the rate of H_2 recovery across all diets and all times, compared to control (table 2.6).

Effects of BCM on SCFA

The data for the effect of BCM on SCFA are summarized in tables 2.7-2.9. At 6 h, total SCFA were not affected by the BCM treatment in the HF and LF diets (tables 2.7 & 2.9) but decreased ($P > 0.01$) in the MF diet (table 2.8). At 12 h, total SCFA significantly decreased with

the inclusion levels of BCM in the LF diet (table 2.9). At 24 h, total SCFA significantly decreased in the MF diet (table 2.8) and tended to decrease ($P > 0.10$) in the LF diet (table 2.9).

There was a significant effect of BCM on both acetate and propionate molar proportions. Irrespective of diets, BCM decreased acetate and increased propionate (tables 2.7-2.9). This resulted in a lower acetate-to-propionate ratio (A:P). At both 12 and 24 h, BCM cultures had a greater butyrate concentration in the MF and HF diets but not the LF diet. There were no effects of BCM on valerate at 6 and 12 h; however, by 24 h, cultures receiving BCM had an increased valerate concentration irrespective of the diet. Addition of BCM to the HF or MF diets had little effect on the isoacid content, but in the LF diet, it resulted in a decreased isoacid concentration.

2.5 Discussion

Complete inhibition of *in-vitro* methanogenesis by BCM

As expected, inclusion of BCM consistently and effectively inhibited CH_4 regardless of diets. The amounts of CH_4 measured in cultures were negligible for all levels of BCM inclusion (figure 2.2). The potent anti-methanogenic effect of BCM observed in the present study is consistent with previous works *in-vitro* and *in-vivo* with BCM alone (Trei & Olson, 1969; Trei et al., 1970; Sawyer et al., 1974) and BCM-CD formulation (McCrabb et al., 1997; Tomkins et al., 2009).

In-vitro and *in-vivo* use of BCM has been associated with a significant decline in the methanogen population, which attest to the specificity of its anti-methanogenic property (Goel et al., 2009; Mitsumori et al., 2012; Denman et al., 2007 & 2015). Denman et al. (2007) found that the vast majority of methanogen amplicons from methyl coenzyme M reductase (*mcrA*) gene was related to *Methanobrevibacter ruminantium* and was inhibited by 0.3 g BCM-CD/100 kg bodyweight inclusion in six Brahman-crossed steers weighing 363±4.7 kg, while methanogen

diversity increased, perhaps due to decreased dominance by *M. ruminantium*. Although microbial composition of samples treated with BCM was not analyzed, a similar reduction in methanogen population most likely occurred due to a high correlation between methanogen population and methanogenic activity (Denman et al., 2007).

Moreover, owing to the functional role of CH_4 as a H_2 sink, CH_4 inhibition by BCM in batch culture (Trei & Olson, 1969; Goel et al., 2009) and *in-vivo* (Abecia et al., 2012; Mitsumori et al., 2012) has been consistently shown to increase H_2 in the gaseous phase. In the present study, peaks for H_2 were observed in GC for BCM treated samples (data not shown). H_2 recovery rate was also lower than control for all inclusion levels of BCM (table 2.6). Due to the lack of methanogenesis as a H_2 sink, the accumulation of H_2 may down-regulate microbial fiber digestion (Wolin, 1979). Nevertheless, experimental results by a few studies suggest that *in-vivo* BCM supplementation does not hinder fiber digestibility and DM intake since not all bacteria are susceptible to H_2 accumulation to some degree (McCrabb et al., 1997; Goel et al., 2009; Tomkins et al., 2009; Mitsumori et al., 2012). The population of ruminal cellulolytic bacterium, *Ruminococcus flavefaciens*, has been suppressed by BCM addition *in vitro* and *in vivo* because *R. flavefaciens* as well as *R. albus* produces H_2 as a by-product of carbohydrate fermentation (Goel et al., 2009; Mitsumori et al., 2012). However, BCM is associated with an increased number of *Fibrobacter succinogenes* (Goel et al., 2009; Mitsumori et al., 2012), which is a major cellulolytic bacterium in the rumen (Stewart & Flint 1989; Shinkai et al., 2009). In fact, because *F. succinogenes* is not a H_2 producer, H_2 accumulation does not affect this bacterial species (Mitsumori et al., 2012, Wolin et al., 1997). In the present study, pH generally decreased at 12 h of incubation in the BCM treatment group (figure 2.3) while total SCFA thereof was unaffected by and large. Pearson analysis shows a significant, negative correlation ($R = -0.89$, $p < 2.2e\text{-}16$)

between pH and total SCFA (figure 2.4) as accumulation of organic acids depresses pH (Aikman et al., 2015). The rise in *F. succinogenes* population may have mediated fiber digestion (Mitsumori et al., 2012).

Although BCM completely inhibited CH_4, it did not seem to shut down fermentation as total SCFA remained fairly high even with the 0.30 g/kg DM inclusion level. However, the decrease in A:P ratio was observed consistently in the present study, which is also in agreement with other studies, (McCrabb et al., 1997; Denman et al., 2007; Goel et al., 2009) and indicative of H_2 redirection towards propionate production (Denman et al., 2007). In addition to *F. succinogenes*, *Prevotella ruminicola* and *Selenomonas ruminantium* population increased in other studies with BCM supplementation (Mitsumori et al., 2012; Denman et al., 2015; Abecia et al., 2018; Matsui et al., 2020). *P. ruminicola* has remarkable resistance to low ruminal pH (Russell & Dombrowski, 1980). In BCM treatment, the genera *Prevotella* and *Selenomonas* may play a primary role in the redirection of H_2 towards propionate production via the succinate pathway (Mitsumori et al., 2012; Denman et al., 2015). Metagenomic analysis of the rumen microbiome in goats fed various BCM doses revealed the absence of acrylate pathway and presence of succinate pathway of propionate production, while enzyme reads involved in the production of propionate via pyruvate in the succinate pathway increased in high dose BCM treatment compared to control (Denman et al., 2015). The shift of SCFA production towards propionate is favorable in ruminant livestock because of its glycogenic property, thereby promoting energy utilization (Schultz & Smith, 1951). Because increased glycogenesis leads to gluconeogenesis in the ruminant, the decline in A:P also has a prophylactic effect on ketotic stress (Prins, 1970).

In the present study, the increase in propionate was concomitant with decreasing acetate, which is consistent with other studies (McCrabb et al., 1997; Mitsumori et al., 2012; Denman et al., 2015). A possible explanation for this observation is altered acetogen community composition (Mitsumori et al., 2014), and a reported increase of an acetogen in the genus *Sporomusa* may be attributed to its unique utilization of lactate and methanol in acetogenesis (Denman et al., 2015). As described above, BCM disrupts cobamide-dependent methyl transferases through its reaction with reduced vitamin B_{12} (Wood et al., 1968), which is involved in both acetoclastic methanogenesis and reductive acetogenesis in the Wood-Ljungdahl pathway (Banerjee & Ragsdale, 2003).

Additionally, as in the present study, increased butyrate production has been reported with BCM treatment (Goel et al., 2009; Sirohi & Goel, 2013). Due to the absence of methanogenesis, spared H_2 as an electron donor was utilized in reduction of NAD and subsequently acetoacetyl-CoA to butyryl-CoA, leading to butyrate production, indicating the abundance of H_2 (Macfarlane & Macfarlane, 2003).

Conclusion

In conclusion, the present study demonstrates the efficacy of BCM on CH_4 inhibition in the rumen. As expected, direct inhibition of a methanogenic enzyme resulted in elevated H_2 accumulation. Microbial analysis on the samples treated with BCM may provide further explanation for the observed effects on methanogenesis and other fermentation parameters. Nevertheless, the experimental result in the present study exemplifies the adaptability of the ruminal fermentation and interspecies H_2 transfer to maximize energy production. Additionally, because BCM is so effective as an anti-methanogenic agent, future investigation should seek a

more environmentally friendly compound with a similar mode of action without ozone depleting potential.

2.6 Tables

Table 2.1. Formulation of three basal diets (high, medium, and low forage) in a 500 g batch used for the experiments

Ingredients	Basal diets		
	High forage (F:C=70:30)	Medium forage (50:50)	Low forage (30:70,)
Alfalfa pellets (F), *g*	350	250	150
Concentrate mix[1] (C), *g*	150	250	350
Ground corn, *g*	119.8	204.4	288.7
Soybean meal, *g*	25.2	40.6	56.3
Vitamin & mineral premix[2], *g*	5.0	5.0	5.0
Zn, *g*	0.06	0.06	0.06
Cu, *mg*	16.9	16.9	16.9
Co, *mg*	0.13	0.13	0.13
I, *mg*	1.30	1.30	1.30
Fe, *mg*	6.50	6.50	6.50
Mn, *mg*	39.0	39.0	39.0
Se, *mg*	0.39	0.39	0.39
Vitamin A, *IU*	8267	8267	8267
Vitamin D_3, *IU*	1102	1102	1102
Vitamin E, *IU*	5.51	5.51	5.51
Total, *g*	500	500	500

[1]Concentrate mix included ground corn, soybean meal, and vitamin and mineral mix.
[2]Vitamin and mineral mix included 1.2% Zn, 3,380 ppm Cu, 26 ppm Co, 260 ppm I, 1,300 ppm Fe, 7,800 ppm Mn, 78 ppm Se, 750,000 IU/lb vitamin A, 100,000 IU/lb vitamin D_3, 5,000 IU/lb vitamin E.

Table 2.2. Chemical composition of three basal diets (high, medium, and low forage) on a dry matter basis (DM)

Chemical composition[1]	Basal diets		
	High Forage (F:C=70:30)	Medium Forage (50:50)	Low Forage (30:70)
Dry matter, *%*	88.8	88.4	88.0
Acid detergent fiber, *%*	27.1	20.7	14.2
Neutral detergent fiber, *%*	35.9	28.8	21.7
Crude protein[2], *%*	18.0	18.1	18.2
Metabolizable energy, *Mcal/kg DM*	2.28	2.35	2.42
Net energy, *Mcal/kg DM*	1.72	1.93	2.15
Ether extract, *%*	3.16	3.46	3.75
Ca, *%*	1.2	0.88	0.57
P, *%*	0.34	0.36	0.38
Mg, *%*	0.28	0.25	0.21
K, *%*	2.10	1.80	1.40
Na, *‰*	0.86	0.68	0.51
Cl, *%*	0.54	0.42	0.29
S, *%*	0.25	0.23	0.20

[1]Chemical composition of each basal diet was calculated from nutrient values obtained in NRC (2001) for alfalfa hay (International Feed #: 1-00-023), ground corn (4-02-854), and soybean meal (5-20-638) unless otherwise stated.
[2]Crude protein contents of ground corn and soybean meal were 16 and 48% DM, respectively.

Table 2.3. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, $p <$	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.34[a]	0.06[b]	0.04[b]	0.03[b]	0.08	0.01	0.01
pH	5.87	5.80	5.87	5.85	0.02	0.98	0.61
NH_3-N, *mg/dL*	8.80	12.32	11.12	11.53	1.75	0.24	0.20
12 hours							
CH_4, *mM*	1.99[a]	0.03[b]	0.03[b]	0.02[b]	0.28	0.01	0.01
pH	5.54[c]	5.51[cd]	5.46[d]	5.48[cd]	0.02	0.05	0.13
NH_3-N, *mg/dL*	8.22	10.36	9.66	10.49	2.14	0.41	0.65
24 hours							
CH_4, *mM*	1.67[a]	0.03[b]	0.02[b]	0.02[b]	0.16	0.01	0.01
pH	5.21[a]	5.32[b]	5.34[b]	5.33[b]	0.01	0.01	0.01
NH_3-N, *mg/dL*	8.92	9.27	12.60	8.20	2.20	0.78	0.05

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 2.4. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, *p* <	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.20[a]	0.06[b]	0.04[b]	0.03[b]	0.09	0.01	0.01
pH	5.82	5.83	5.87	5.87	0.02	0.08	0.47
NH_3-N, *mg/dL*	9.48	10.58	11.58	11.86	1.56	0.26	0.62
12 hours							
CH_4, *mM*	2.29[a]	0.04[b]	0.02[b]	0.02[b]	0.24	0.01	0.01
pH	5.52[a]	5.46[a]	5.43[b]	5.39[b]	0.03	0.01	0.33
NH_3-N, *mg/dL*	11.18	9.79	9.22	10.37	1.13	0.66	0.17
24 hours							
CH_4, *mM*	1.82[a]	0.02[b]	0.01[b]	0.02[b]	0.17	0.01	0.01
pH	5.21	5.24	5.24	5.21	0.01	0.98	0.02
NH_3-N, *mg/dL*	8.41	10.03	8.74	9.62	1.76	0.45	0.72

[a, b]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 2.5. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, *p* <	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
CH_4, *mM*	1.49[a]	0.06[b]	0.04[b]	0.03[b]	0.04	0.01	0.01
pH	5.87	5.85	5.86	5.90	0.02	0.12	0.12
NH_3-N, *mg/dL*	9.40[a]	12.46[bc]	11.33[b]	13.70[c]	1.11	0.02	0.56
12 hours							
CH_4, *mM*	1.92[a]	0.02[b]	0.02[b]	0.02[b]	0.05	0.01	0.01
pH	5.50[a]	5.31[b]	5.28[b]	5.38[c]	0.03	0.01	0.01
NH_3-N, *mg/dL*	7.57	6.69	7.25	7.29	1.65	0.99	0.78
24 hours							
CH_4, *mM*	2.50[a]	0.03[b]	0.01[b]	0.01[b]	0.22	0.01	0.01
pH	5.12[a]	5.18[bc]	5.20[c]	5.16[ab]	0.02	0.13	0.01
NH_3-N, *mg/dL*	8.29	7.73	6.02	5.48	2.06	0.29	0.65

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 2.6. Effect of 3 levels (0.075, 0.15, 0.30 g/kg DM) of bromochloromethane (BCM) on hydrogen (H_2) recovery rate[1] in mixed cultures of rumen microbes fed three basal diets[2] varying in forage to concentrate[3] ratio (F:C) and incubated at 39°C for 6, 12, and 24 hours.

		H_2 recovery, *%*						
		BCM, *g/kg DM*					Trend, *p* <	
		0	0.075	0.15	0.30	SEM[4]	Linear	Quadratic
Basal diets (F:C)	***6 hours***							
	High forage (70:30)	78.0[a]	41.7[b]	40.8[b]	40.6[b]	4.47	0.01	0.01
	Medium forage (50:50)	70.9[a]	44.3[b]	42.5[b]	42.0[b]	2.32	0.01	0.01
	Low forage (30:70)	80.6[a]	43.0[b]	40.3[b]	40.0[b]	1.38	0.01	0.01
	12 hours							
	High forage (70:30)	81.8[a]	42.5[b]	43.4[b]	44.2[b]	3.94	0.01	0.01
	Medium forage (50:50)	93.9[a]	45.7[b]	45.2[b]	45.0[b]	4.49	0.01	0.01
	Low forage (30:70)	84.5[a]	45.4[b]	45.3[b]	45.5[b]	1.09	0.01	0.01
	24 hours							
	High forage (70:30)	71.8[a]	45.9[b]	46.3[b]	46.6[b]	3.02	0.01	0.01
	Medium forage (50:50)	75.3[a]	47.6[b]	48.0[b]	48.8[b]	3.36	0.01	0.01
	Low forage (30:70)	89.9[a]	48.1[b]	48.2[b]	49.2[b]	4.25	0.01	0.01

[a, b]different superscripts within a row differ ($p \leq 0.05$)

[1]H_2 recovery rate was calculated by:

$$H_2\ recovery\ (\%) = \left(\frac{4 \times CH_4 + 2 \times Propionate + 2 \times Butyrate + Valerate}{2 \times Acetate + Propionate + 4 \times Butyrate + 2 \times Isobutyrate + 2 \times Valerate}\right) \times 100$$

(Wolin, 1960; Van Soest, 1994; Blümmel et al., 1997)

[2]Three basal diets were high forage (F:C=70:30), medium forage (50:50), and low forage (30:70)

[3]Concentrate consisted of corn and soybean meal.

[4]Standard error of the mean

Table 2.7. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, *p* <	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.06	100.03	100.29	100.15	9.62	0.91	0.92
Individual SCFA	*mol %*						
Acetate (A)	64.27[a]	60.80[b]	60.78[b]	60.89[b]	0.69	0.02	0.02
Propionate (P)	23.98[a]	28.26[b]	27.91[b]	27.70[b]	0.47	0.01	0.01
Butyrate	9.68	9.12	9.46	9.51	0.64	1.00	0.59
Isobutyrate	0.44	0.38	0.37	0.37	0.07	0.53	0.66
Valerate	0.96	0.86	0.92	0.96	0.11	0.83	0.59
Isovalerate	0.68	0.57	0.55	0.56	0.08	0.36	0.43
A:P Ratio	2.69[a]	2.15[b]	2.18[b]	2.20[b]	0.05	0.01	0.01
12 hours							
Total SCFA, *mM*	128.86	126.50	127.85	139.37	4.23	0.08	0.21
Individual SCFA	*mol %*						
Acetate (A)	61.79[a]	58.24[b]	57.28[b]	56.56[b]	1.10	0.01	0.11
Propionate (P)	26.01[d]	28.65[de]	29.21[e]	29.93[e]	1.34	0.03	0.21
Butyrate	10.23[d]	11.24[de]	11.63[e]	11.64[e]	1.02	0.04	0.12
Isobutyrate	0.41[d]	0.28[e]	0.28[e]	0.29[e]	0.04	0.09	0.07
Valerate	1.01	1.16	1.20	1.18	0.17	0.17	0.17
Isovalerate	0.55	0.42	0.40	0.40	0.05	0.08	0.11
A:P Ratio	2.45[d]	2.03[d]	1.96[e]	1.90[e]	0.14	0.03	0.14
24 hours							
Total SCFA, *mM*	152.92	126.88	131.79	147.01	9.83	0.97	0.05
Individual SCFA	*mol %*						
Acetate (A)	57.95[a]	51.92[b]	51.55[b]	51.12[b]	1.19	0.01	0.02
Propionate (P)	26.06[a]	28.23[b]	29.04[bc]	29.39[c]	0.66	0.01	0.01
Butyrate	14.07	17.52	17.20	17.12	1.35	0.15	0.11
Isobutyrate	0.32	0.28	0.26	0.31	0.03	0.97	0.13
Valerate	1.08[d]	1.58[e]	1.51[e]	1.55[e]	0.21	0.06	0.08
Isovalerate	0.52	0.47	0.44	0.50	0.04	0.79	0.13
A:P Ratio	2.23[a]	1.84[b]	1.77[b]	1.74[b]	0.05	0.01	0.01

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 2.8. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, *p* <	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	102.84[a]	93.82[a]	83.09[b]	78.29[b]	3.38	0.01	0.12
Individual SCFA	*mol %*						
Acetate (A)	62.86[a]	57.62[b]	58.92[b]	59.39[b]	0.73	0.03	0.01
Propionate (P)	24.78[a]	29.03[b]	28.42[b]	28.20[b]	1.05	0.01	0.01
Butyrate	10.24	11.18	10.64	10.47	0.55	0.93	0.20
Isobutyrate	0.40[d]	0.34[de]	0.33[de]	0.29[e]	0.03	0.02	0.56
Valerate	1.05	1.17	1.15	1.10	0.08	0.70	0.08
Isovalerate	0.66	0.66	0.54	0.56	0.06	0.21	0.54
A:P Ratio	2.56[a]	2.00[b]	2.08[b]	2.11[b]	0.11	0.01	0.01
12 hours							
Total SCFA, *mM*	118.34	111.33	117.07	118.23	4.72	0.57	0.28
Individual SCFA	*mol %*						
Acetate (A)	60.48[a]	55.47[b]	54.98[b]	54.82[b]	0.58	0.01	0.01
Propionate (P)	25.74[a]	30.35[b]	29.86[b]	29.45[b]	1.06	0.01	0.01
Butyrate	11.75[a]	12.29[ab]	13.28[bc]	13.87[c]	0.94	0.01	0.41
Isobutyrate	0.36	0.26	0.27	0.25	0.04	0.09	0.21
Valerate	1.13	1.20	1.19	1.21	0.09	0.20	0.53
Isovalerate	0.52[a]	0.43[b]	0.42[b]	0.40[b]	0.02	0.01	0.11
A:P Ratio	2.39[a]	1.83[b]	1.84[b]	1.86[b]	0.10	0.01	0.01
24 hours							
Total SCFA, *mM*	156.83[a]	135.15[b]	135.51[b]	135.74[b]	5.13	0.02	0.02
Individual SCFA	*mol %*						
Acetate (A)	55.26[a]	49.01[b]	48.08[b]	47.47[b]	1.92	0.01	0.04
Propionate (P)	26.83[a]	29.11[b]	29.18[bc]	29.78[c]	1.03	0.01	0.01
Butyrate	16.05[d]	19.71[de]	20.54[e]	20.43[e]	2.08	0.06	0.10
Isobutyrate	0.34	0.29	0.26	0.29	0.03	0.24	0.11
Valerate	1.01[a]	1.43[b]	1.49[b]	1.55[b]	0.23	0.01	0.05
Isovalerate	0.51	0.45	0.44	0.47	0.04	0.63	0.28
A:P Ratio	2.06[a]	1.68[b]	1.64[b]	1.59[b]	0.05	0.01	0.01

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

Table 2.9. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane (BCM) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	BCM, *g/kg DM*					Trend, *p* <	
	0	0.075	0.15	0.30	SEM[2]	Linear	Quadratic
6 hours							
Total SCFA, *mM*	104.39	88.91	79.68	86.61	10.29	0.24	0.17
Individual SCFA	*mol %*						
Acetate (A)	60.56[a]	58.37[b]	61.06[a]	61.06[a]	0.50	0.13	0.06
Propionate (P)	23.78	27.37	35.11	26.63	5.28	0.68	0.20
Butyrate	11.41	12.36	13.00	10.32	1.80	0.64	0.38
Isobutyrate	0.55[ab]	0.30[a]	0.91[b]	0.55[a]	0.14	0.94	0.21
Valerate	1.21	1.06	1.51	1.00	0.18	0.62	0.25
Isovalerate	0.85[ab]	0.53[a]	0.99[b]	0.58[a]	0.10	0.31	0.37
A:P Ratio	2.52[a]	2.15[b]	2.22[bc]	2.30[c]	0.09	0.08	0.01
12 hours							
Total SCFA, *mM*	121.80[a]	114.43[ab]	108.04[b]	110.36[b]	2.72	0.02	0.04
Individual SCFA	*mol %*						
Acetate (A)	58.47[a]	52.85[b]	52.79[b]	52.64[b]	0.48	0.01	0.01
Propionate (P)	25.52[a]	28.48[b]	28.35[b]	28.51[b]	0.74	0.04	0.06
Butyrate	13.88[a]	16.73[b]	17.01[b]	16.87[b]	0.48	0.01	0.01
Isobutyrate	0.39[a]	0.28[b]	0.25[b]	0.30[b]	0.02	0.13	0.01
Valerate	1.16	1.22	1.19	1.24	0.03	0.12	0.72
Isovalerate	0.58[a]	0.45[b]	0.40[b]	0.43[b]	0.03	0.02	0.01
A:P Ratio	2.32[a]	1.86[b]	1.86[b]	1.85[b]	0.08	0.01	0.02
24 hours							
Total SCFA, *mM*	147.76[d]	128.97[e]	140.36[de]	137.60[de]	4.09	0.36	0.12
Individual SCFA	*mol %*						
Acetate (A)	49.37	48.08	46.58	46.01	2.29	0.06	0.46
Propionate (P)	26.13[a]	29.53[b]	28.58[b]	29.62[b]	1.20	0.01	0.06
Butyrate	22.26[d]	20.25[e]	22.42[d]	22.00[de]	2.54	0.55	0.42
Isobutyrate	0.38[a]	0.27[b]	0.30[b]	0.30[b]	0.02	0.02	0.01
Valerate	1.23	1.31	1.70	1.60	0.30	0.08	0.26
Isovalerate	0.62[a]	0.40[b]	0.42[b]	0.47[b]	0.03	0.03	0.01
A:P Ratio	1.90[a]	1.61[b]	1.63[b]	1.55[b]	0.08	0.02	0.12

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]Standard error of the mean

2.7 Figures

Figure 2.1. Simplified experimental design of *in-vitro* fermentation per level of BCM inclusion (0, 0.075, 0.15, & 0.30 g/kg DM) in a batch run.

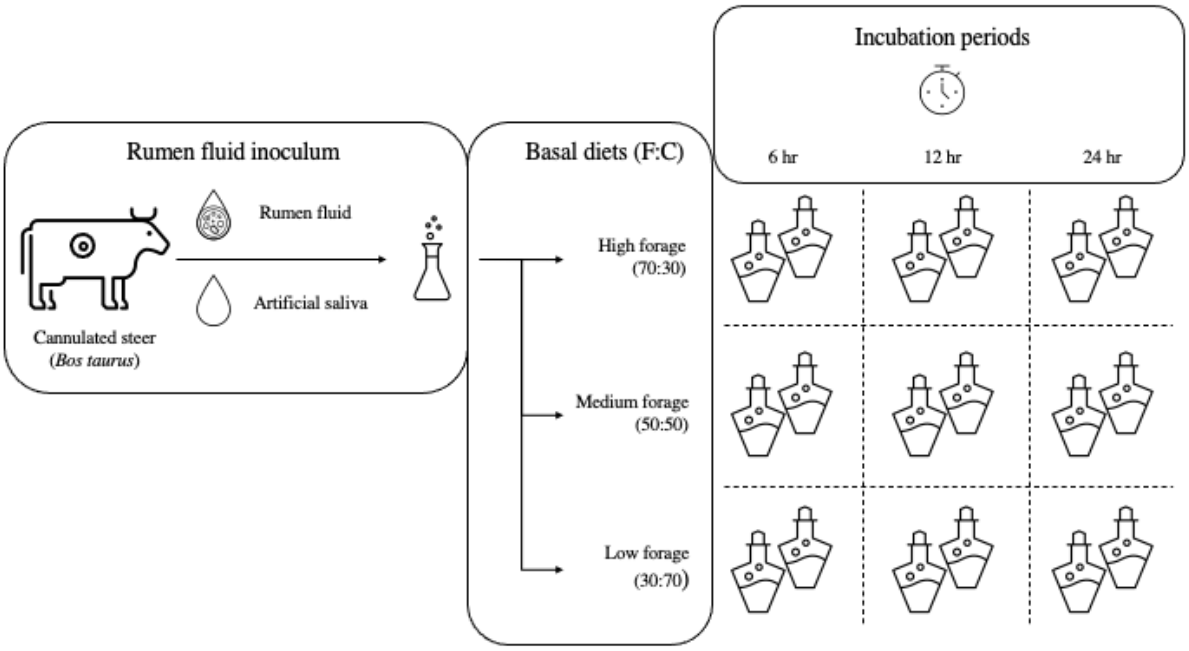
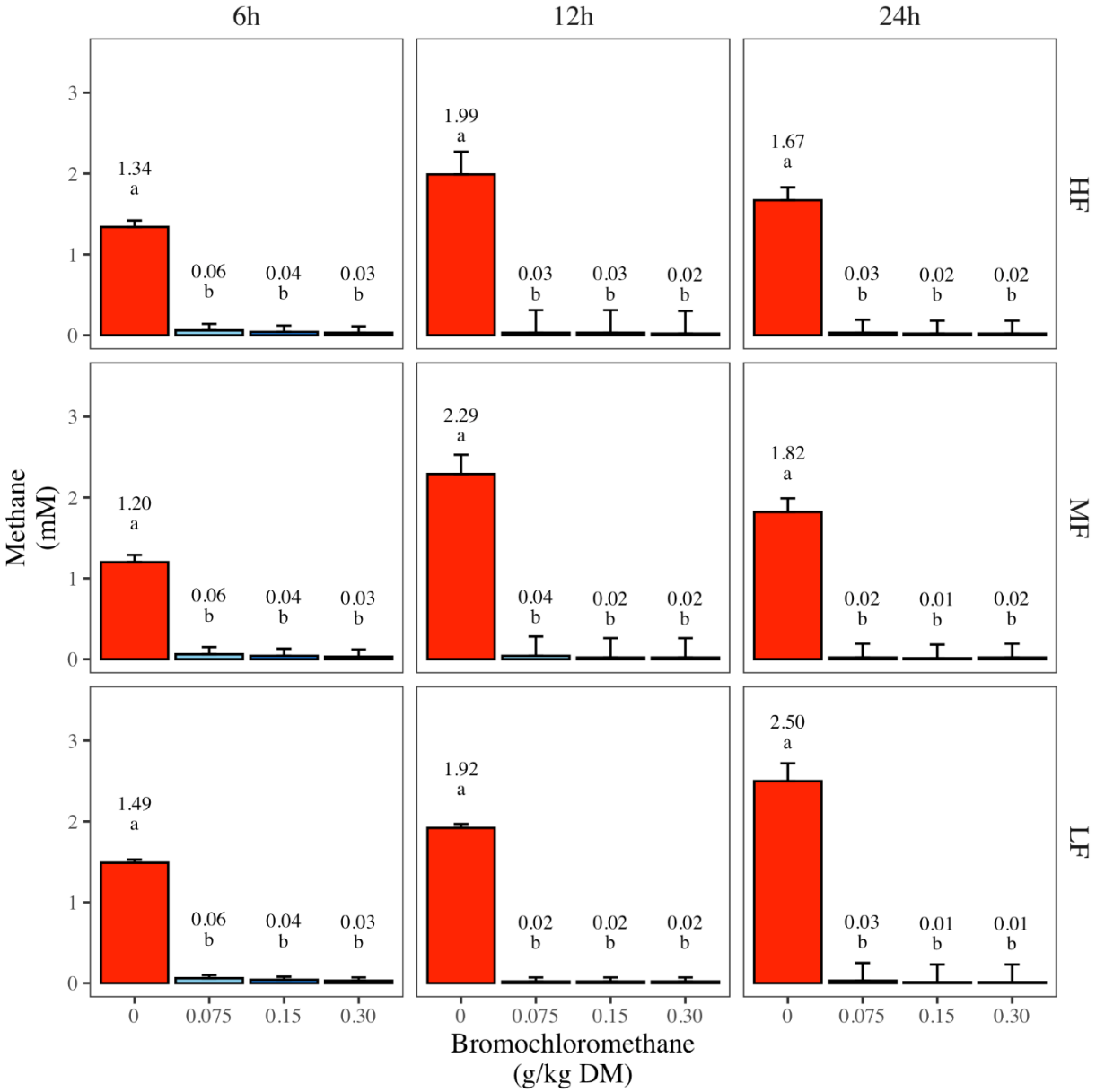


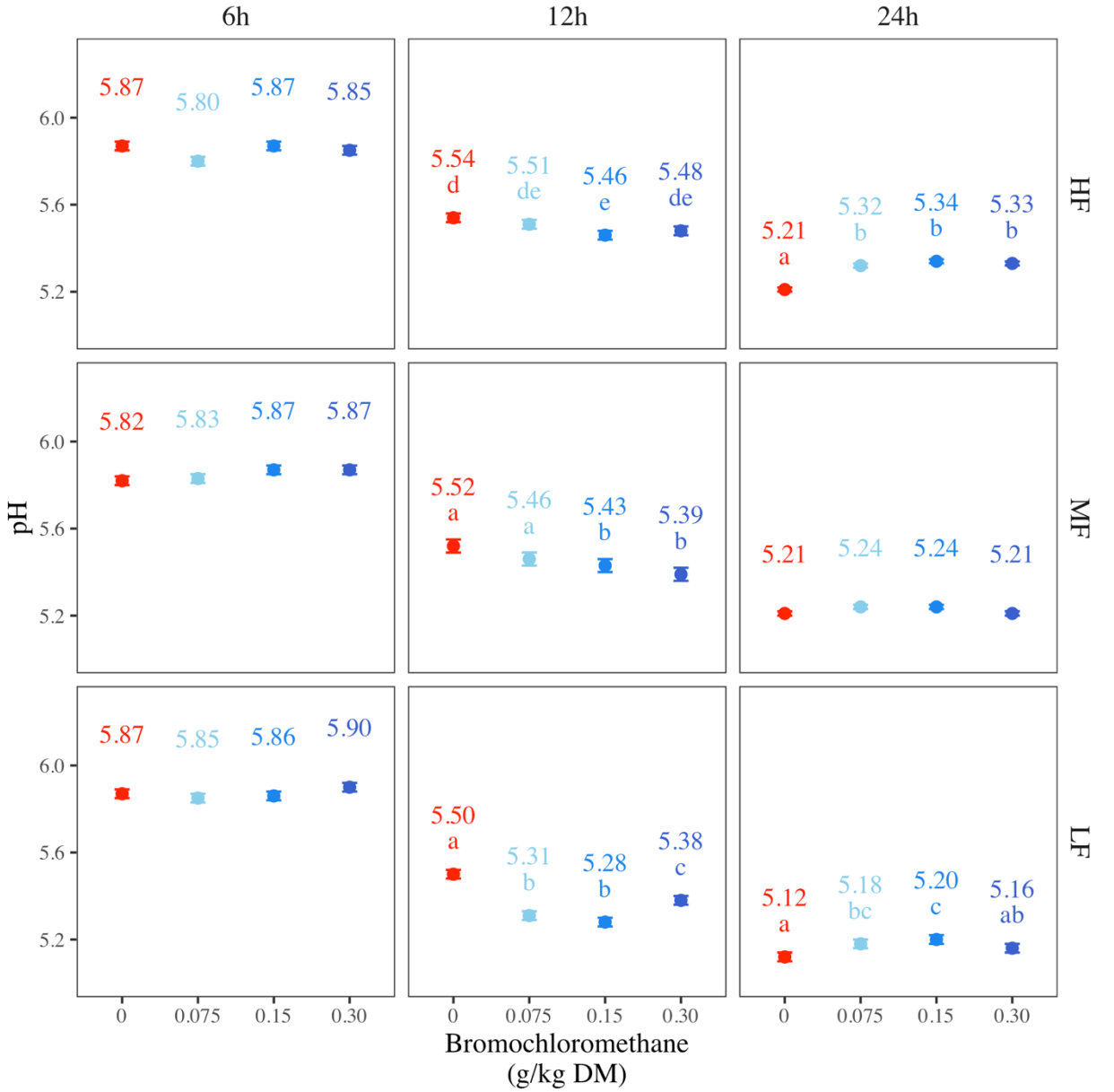
Figure 2.2. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane on methane in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a, b]different superscripts within a row differ ($p \leq 0.05$)

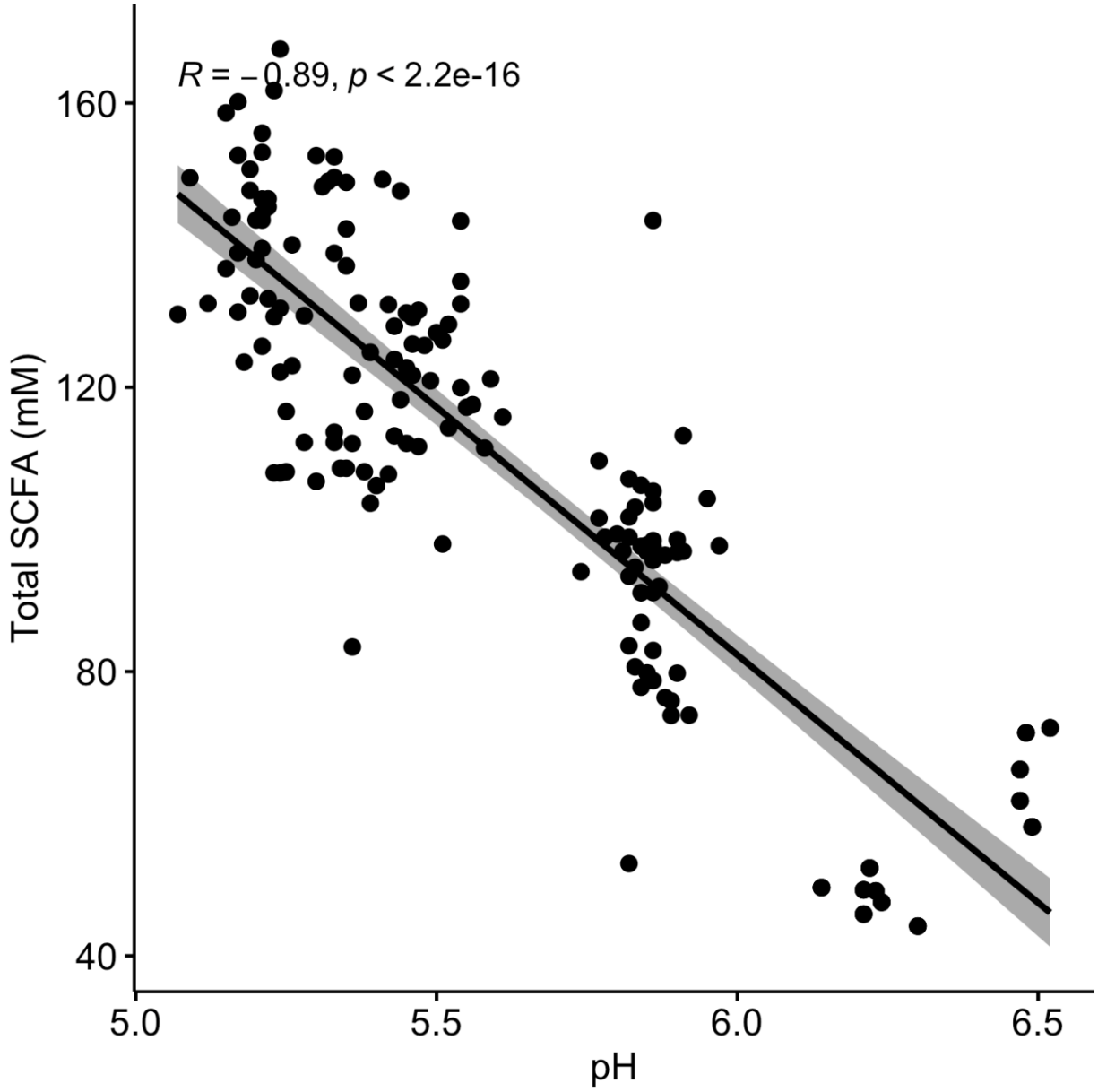
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 2.3. Effect of 3 levels (0.075, 0.15, and 0.30 g/kg DM) of bromochloromethane on pH in mixed cultures of rumen microbes fed diets[1] varying in forage-to-concentrate ratio and incubated at 39°C for 6, 12, and 24 hours.



[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 2.4. Pearson correlation between pH and total short-chain fatty acid (SCFA; mM) at the bromochloromethane (BCM) inclusion levels 0, 0.075, 0.15, and 0.30 g/kg DM



REFERENCE

Abecia, L., Toral, P. G., Martín-García, A. I., Martínez, G., Tomkins, N. W., Molina-Alcaide, E., Newbold, C. J., & Yáñez-Ruiz, D. R. (2012). Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. *Journal of dairy science*, *95*(4), 2027–2036. https://doi.org/10.3168/jds.2011-4831

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J., & Yáñez-Ruiz, D. R. (2018). Analysis of the Rumen Microbiome and Metabolome to Study the Effect of an Antimethanogenic Treatment Applied in Early Life of Kid Goats. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/articles/10.3389/fmicb.2018.02227

Aikman, P. C., Henning, P. H., Humphries, D. J., & Horn, C. H. (2011). Rumen pH and fermentation characteristics in dairy cows supplemented with Megasphaera elsdenii NCIMB 41125 in early lactation. *Journal of Dairy Science*, *94*(6), 2840–2849. https://doi.org/10.3168/jds.2010-3783

Beecher, G. R., & Whitten, B. K. (1970). Ammonia determination: Reagent modification and interfering compounds. *Analytical Biochemistry*, *36*(1), 243–246. https://doi.org/10.1016/0003-2697(70)90356-8

Blaxter, K., & Clapperton, J. (1965). Prediction of the amount of methane produced by ruminants. *British Journal of Nutrition, 19*(1), 511-522. doi:10.1079/BJN19650046

Blümmel, M., Makkar, H. P. S., & Becker, K. (1997). In vitro gas production: A technique revisited. *Journal of Animal Physiology and Animal Nutrition*, *77*(1–5), 24–34. https://doi.org/10.1111/j.1439-0396.1997.tb00734.x

Chalupa, W. (1977). Manipulating Rumen Fermentation, *Journal of Animal Science*, *45*(3), 585–599, https://doi.org/10.2527/jas1977.453585x

Denman, S. E., Tomkins, N. W., & McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane, *FEMS Microbiology Ecology*, *62*(3), 313–322, https://doi.org/10.1111/j.1574-6941.2007.00394.x

Denman, S. E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., & McSweeney, C. S. (2015). Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. *Frontiers in Microbiology*, *6*, 1087. https://doi.org/10.3389/fmicb.2015.01087

EPA (2022) Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2020. U.S. Environmental Protection Agency, EPA 430-R-22-003. https://www.epa.gov/ghgemissions/draft-inventory-us-greenhouse-gas-emissionsand-sinks-1990-2020.

Goel, G., Makkar, H. P., & Becker, K. (2009). Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. *The British Journal of Nutrition*, *101*(10), 1484–1492. https://doi.org/10.1017/S0007114508076198

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science*, *73*(8), 2483–2492. https://doi.org/10.2527/1995.7382483x

Kassambra, A. (2020). ggpubr: *'ggplot2' Based Publication Ready Plots. R package version 0.4.0, <https://CRAN.R-project.org/package=ggpubr>.*

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *The Proceedings of the Nutrition Society*, *62*(1), 67–72. https://doi.org/10.1079/PNS2002207

Matsui, H., Tajima, K., & Itabashi, H. (2020). Diversity of Prokaryotes in the Rumen of Steers Fed a Diet Supplemented with or without Bromochloromethane, an Anti-methanogenic Compound. *Japan Agricultural Research Quarterly*, *54*(2), 179–183. https://doi.org/10.6090/jarq.54.179

May, C., Payne, A. L., Stewart, P. L., and Edgar, J. A. (1996). A delivery system for antimethanogenic agents. International Patent Application No. PCT/AU95/00733.

McCrabb, G. J., Berger, K. T., Magner, T., May, C. & Hunter, R. A. (1997). Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. *Australian Journal of Agricultural Research*, *48*, 323-329.

McDougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *The Biochemical Journal*, *43*(1), 99–109.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., & McSweeney, C. S. (2012). Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *The British Journal of Nutrition*, *108*(3), 482–491. https://doi.org/10.1017/S0007114511005794

Mitsumori, M., Matsui, H., Tajima, K., Shinkai, T., Takenaka, A., Denman, S. E., & McSweeney, C. S. (2014). Effect of bromochloromethane and fumarate on phylogenetic diversity of the formyltetrahydrofolate synthetase gene in bovine rumen. *Animal science journal = Nihon chikusan Gakkaiho*, *85*(1), 25–31. https://doi.org/10.1111/asj.12072

National Research Council. (2001). *Nutrient Requirements of Dairy Cattle: Seventh Revised Edition, 2001*. Washington, DC: The National Academies Press. https://doi.org/10.17226/9825.

Paynter, M. J., & Elsden, S. R. (1970). Mechanism of propionate formation by Selenomonas ruminantium, a rumen micro-organism. *Journal of General Microbiology*, *61*(1), 1–7. https://doi.org/10.1099/00221287-61-1-1

Prins R. A. (1970). Methanogenesis and propionate production in the rumen as influenced by therapeutics against ketosis. *Zeitschrift fur Tierphysiologie, Tierernahrung und Futtermittelkunde*, *26*(3), 147–151. https://doi.org/10.1111/j.1439-0396.1970.tb00135.x

Sawyer, M. S., Hoover, W. H., & Sniffen, C. J. (1974). Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. *Journal of Animal Science*, *38*(4), 908–914. https://doi.org/10.2527/jas1974.384908x

Schultz, L. H., & Smith, V. E. (1951). Experimental Alteration of the Sugar and Ketone Levels of the Blood of Ruminants in Relation to Ketosis1, 2. *Journal of Dairy Science*, *34*(12), 1191–1199. https://doi.org/10.3168/jds.S0022-0302(51)91847-4

Shinkai, T., Ohji, R., Matsumoto, N., & Kobayashi, Y. (2009). Fibrolytic capabilities of ruminal bacterium Fibrobacter succinogenes in relation to its phylogenetic grouping. *FEMS Microbiology Letters*, *294*(2), 183–190. https://doi.org/10.1111/j.1574-6968.2009.01565.x

Sirohi, S., & Goel, N. (2013). Effect of various levels of bromochloromethane and fibre level on in vitro methane production and fermentation pattern. *The Indian Journal of Animal Science*, *83*(8), 798-801.

Tomkins, N. W. & Hunter, R. A. (2004). Methane reduction in beef cattle using a novel antimetahnogen. *Animal Production in Australia*, *25*, 329.

Tomkins, N. W., Colegate, S. M., & Hunter, R. A. (2009). A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. *Animal Production Science*, *49*(12), 1053-1058.

Trei, J. E. & Olson, W. A. (1969). Effect of chlorine containing analogues of methane on rumen fermentation. *Journal of Animal Science*, *29*, 173-174.

Trei, J. E., Singh, Y. K., & Scott, G. C. (1970). Effect of methane inhibitors on rumen metabolism. *Journal of Animal Science*, *31*, 392.

Van Soest, P. J. (1994). *Nutritional ecology of the ruminant*. Cornell University Press.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Wolin, M. J. (1960). A Theoretical Rumen Fermentation Balance. *Journal of Dairy Science*, *43*(10), 1452–1459. https://doi.org/10.3168/jds.S0022-0302(60)90348-9

Wolin, M. J. (1979). The Rumen Fermentation: A Model for Microbial Interactions in Anaerobic Ecosystems. In: Alexander, M. (eds) Advances in Microbial Ecology. *Advances in Microbial Ecology*, *3*. https://doi.org/10.1007/978-1-4615-8279-3_2

Wolin, M. J., Miller, T. L., and Stewart, C. S. (1997). Microbe-microbe interactions. *The Rumen Microbial Ecosystem*, eds P. N. Hobson and C. S. Stewart (London: Blackie), 467–491. doi: 10.1007/978-94-009-1453-7_11

Wood, J. M., Kennedy, D. S., & Wolfe, R. S. (1968). Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry*, *7*(5), 1707-1713. https://doi.org/10.1021/bi00845a013

CHAPTER 3: Effect of combination of thermodynamic and kinetic inhibitory strategies on ruminal methanogenesis

3.1 Abstract

Methane (CH_4) emission from the ruminant livestock represents a nutritional and environmental fault; potential dietary energy is belched out as CH_4, which is a greenhouse gas contributing to global warming. However, CH_4 serves as a primary hydrogen (H_2) sink in the rumen, which is essential for functional fermentation in the rumen by maintaining low H_2 concentration. Therefore, the abatement strategies of methanogenesis must be concomitant with provision of alternative H_2 sinks. In the present study, we investigated the effect of the most efficacious anti-methanogenic compound, bromochloromethane (0.30 g/kg BCM, B), and 3 alternative H_2 sinks, sodium nitrate (28g/kg $NaNO_3$, N), sodium sulfate (12 g/kg $NaSO_4$, S), and 3-nitro-1-propionate (2.0 g/kg 3NPA, P) on *in-vitro* ruminal fermentation when added to basal diets differing in energy levels.

The experimental design included combination of NS, NSP, and NSPB treatments on high (HF, 70:30 forage to concentrate, F:C), medium (MF, 50:50 F:C), and low (LF, 30:70 F:C) forage diets. Glass bottles (100-mL) with septum caps contained each combination of BCM and basal diet and were inoculated with 30 mL of rumen inoculum from a cannulated Hereford steer. After 6 and 24 h of incubation at 39°C, *in-vitro* fermentation was terminated by placing the culture bottles in ice. Headspace gas samples were analyzed for CH_4, and culture contents were analyzed for pH, NH_3-N, and SCFAs.

After 24 h, CH_4 was inhibited by up to 85, 94, and 99% of the control value in the NS, NSP, and NSPB treatments, respectively. Ammonia-N and propionate were elevated in all treatments compared to the control. The inclusion of BCM in the NSPB treatment exerted

marked differences in fermentation parameters compared to the control and other treatments; in addition to the increases in NH_3-N and propionate, butyrate and valerate were also elevated compared to the NS and NSP treatment while acetate decreased compared to the control. The flow of H_2 represented in the fermentation pattern observed in the NSPB treatment shifted towards propionate, butyrate, valerate, and ammonia, which may lead to a beneficial outcome in terms of animal performance and production.

3.2 Introduction

The marvel of the ruminant digestive physiology resides in the rumen, where anaerobic microorganisms ferment plant carbohydrates such as cellulose and starch into organic acids, namely short-chain fatty acids (Wolin, 1979). In turn, SCFAs are absorbed across the rumen and nourish the ruminant (Wolin, 1979; Van Soest, 1994). Methane (CH_4) is generated as a by-product of ruminal fermentation because the anaerobic nature of the rumen fosters an abundance of reducing equivalents in the form of hydrogen (H_2; Ungerfeld, 2020). In such a highly reducing environment, methanogenesis serves as a primary H_2 sink and thus prevents feedback inhibition of ruminal fermentation by the accumulation of H_2 (Johnson & Johnson, 1995).

However, CH_4 is considered as a flaw from the perspective of feed conversion efficiency and environmental concerns. CH_4 represents an energy loss of 2-10% from the feed escaping as gas (Blaxter and Clapperton, 1965). Then, CH_4 accumulates in the lower atmosphere and causes a warming phenomenon known as the greenhouse effect, hence a greenhouse gas (GHG; Beerling et al., 2009). To improve feed efficiency and to alleviate the environmental impact of ruminant livestock production, research has sought strategies to abate ruminal methanogenesis through dietary means (Latham et al., 2016).

Given the idea that the flow of H_2 serves as the main operator of ruminal fermentation (Morgavi et al., 2018; Ungerfeld, 2020), there are two primary strategies of methanogenesis inhibition that are applicable in forms of feed additives: redirection of H_2 flow from methanogenesis by promoting/introducing H_2 sinks that are thermodynamically competitive to methanogenesis; and blocking the H_2 flow towards methanogenesis by inhibiting an enzyme involved in the methanogenic pathway. The examples of the former are nitrate (NO_3^-) and sulfate (SO_4^{2-}), whose reduction is thermodynamically more favorable than methanogenesis in theory (Thauer et al., 1977). The latter strategy is considered the most effective (Chalupa, 1977) and involves halogenated methane analogues such as bromochloromethane (BCM), which directly inhibits cobamide-dependent methyl transferase (Wood et al., 1968). Despite the efficacious anti-methanogenic effect of BCM, the disadvantage of this strategy is the accumulation of H_2 as a result of major H_2 sink absence shown in batch culture and *in-vivo* experiments (Trei et al., 1970; Goel et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012).

Therefore, we investigated the effects of combinations of kinetic and thermodynamic inhibitors on methanogenesis. The effect of combination of a direct CH_4 inhibitor and multiple H_2 sinks on fermentation pattern is of additional interest. The present study included the combination of sodium nitrate ($NaNO_3$), sodium sulfate ($NaSO_4$), and 3-nitro-1-propionate (3NPA) as H_2 sinks and BCM as a direct CH_4 inhibitor. Additionally, these additives were combined with 3 basal diets to investigate the effect of energy levels on their anti-methanogenic effects.

3.3 Material and Methods

Feed Additives and Feed Substrates

To test the anti-methanogenic effect of combination of NO_3^- (N), SO_4^{2-} (S), 3NPA (P), and BCM (B), experiments were designed to include combination of NS, NSP, and NSPB and three different diets in *in-vitro* batch cultures of mixed rumen microbes. The additive treatment included a control (no additive), 28 g/kg DM of $NaNO_3$, 12 g/kg DM of $NaSO_4$, 2.0 g/kg DM of 3NPA, and 0.30 g/kg DM of BCM. The three diets were formulated with alfalfa hay and concentrate mix for the following F:C: 70:30 for high forage (HF); 50:50 for medium forage (MF); and 30:70 for low forage (LF). The concentrate mix consisted of a mixture of ground corn, soybean meal, and vitamin and mineral mix. The ingredients and chemical composition of the basal diets are summarized in tables 3.1 and 3.2, respectively. Each experimental diet (approx. 1.0 g) was quantitatively weighed in a 100-mL glass culture bottle, in duplicates, and incubated for 6 and 24 h at 39°C. The experiment was replicated twice (n=2) for statistical analysis (figure 3.1).

Rumen Fluid Collection and Inoculation

Rumen inoculum was prepared by mixing rumen fluid and artificial saliva in a 1:2 ratio. The rumen fluid was collected from a cannulated Hereford steer (*Bos taurus*), whose basal diet was a total mixed ration on a perennial grass pasture at the NCSU Metabolic Unit. The artificial saliva was prepared according to the ruminant salivary composition by McDougall (1948) and consisted of $NaHCO_3$, $NaH_2PO_4 \bullet H_2O$, NaCl, KCl, $CaCl \bullet 2H_2O$, $MgCl_2 \bullet 6H_2O$, and Urea. Thirty mL of rumen inoculum were allocated to each fermentation bottle containing 1.0 g of feed substrate and each level of a feed additive while the bottle was flushed with a stream of CO_2 prior to and during inoculation to maintain anaerobicity. Immediately after the inoculation, the

bottles were sealed with rubber-lined septum caps and placed in a water bath at 39ºC for 6 and 24 h. After each time period, respective bottles were transferred to ice bath to terminate the *in-vitro* microbial activity.

Analytical Measurements

The present study measured CH_4, pH, ammonia-N (NH_3-N), and short-chain fatty acids (SCFAs) at 6 and 24 h of *in-vitro* fermentation. Methane concentration in the fermentation bottle was measured using a gas chromatography (model CP-3800; Varian, Walnut Creek, CA) using a stainless-steel column packed with Molsieve 5A 45/60 mesh (Supelco Inc., Bellefonte, PA). Gas samples were withdrawn from the headspace (70-mL) within each bottle with an air-tight syringe (Hamilton Co., Reno, NV). The rubber-lined septum cap retained the gas within the headspace while taking samples.

After CH_4 measurement, pH of the culture fluid in the bottle was measured with a pH probe (VWR SympHony – model AR25; Accumet Research, Dual Channel pH/Ion Meter Fisher Scientific). Following pH measurements, culture contents were transferred to a tube and centrifuged at 2,000 rpm for 5 minutes to separate the solid digesta from the liquid. Four mL of supernatant were aliquoted to a 5.0 mL centrifuge tube and was kept in a freezer at - 20ºC for subsequent NH_3-N and SCFA analyses. After thawing the 4.0-mL of rumen fluid, two 1.0-mL aliquots of culture fluids were transferred into separate microcentrifuge tubes for NH_3-N and SCFA analyses.

NH_3-N concentration was calculated using the colorimetric procedure outlined by Beecher and Whitten (1970). Standards containing 0, 4, 8, 12, and 16 μg/mL of NH_3-N were prepared. Samples were centrifuged at 15,000 rpm for 15 minutes to separate any remaining solid particles from the liquid. For the analysis, 5.0 μL of each sample or standards were

transferred in duplicates into glass tubes. One hundred μL of DI water, and 0.5 mL of phenol and sodium hypochlorite reagents were added. The samples and standards were allowed to react for 30 minutes at room temperature. Following 30 min, 4.0 mL of DI water were added to the sample mixture. The sample was then transferred to a cuvette for absorbance measured at wavelength of 630 nm. The standards were used to determine the concentration of unknown samples.

Concentration of SCFA was measured by gas chromatography (model CP- 3380; Varian, Walnut Creek, CA) using a fused silica capillary column (Nukol^{TM}; Superlco Inc., Bellefonte, PA). One mL aliquots of culture contents were frozen, thawed, and centrifuged at 15,000 rpm for 15 minutes to separate remaining solid particle from the liquid. The 1.0-mL sample aliquot was treated with 0.2 mL of a metaphosphoric acid, which included 2-ethylbutyrate as internal standard. The sample was then centrifuged at 15,000 rpm for 5 minutes and transferred to a GC vial. The column used in this study detected lactate, acetate, propionate, butyrate, valerate, and the isoacids (isobutyrate and isovalerate).

Statistical Analysis

Data from above measurements were analyzed according to a completely randomized block design using the Mixed procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Data from 6 and 24 h were analyzed separately. The model included the main effect of additive levels. Orthogonal contrasts were used to determine linear or quadratic trends. The replicate variable nested within the batch run was treated as a random effect. Thus, the model is represented by:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(k)} + \varepsilon_{ijk}$$

where

y_{ijk} = each response variable measured,

μ = overall mean,

α_i = fixed effect of treatment

$\beta_{j(k)}$ = random effect of replicate nested within run

ε_{ijk} = subplot error.

Significant effects were declared at *p-value* ≤ 0.05 and tendencies at ≤ 0.10. The analyzed data were visualized in figures using the following R packages: ggplot2 (Wickham, 2016) and ggpubr (Kassambra, 2020).

3.4 Results

Effect of combined treatment with $NaNO_3$, $NaSO_4$, 3NPA, and BCM on *in-vitro* fermentation parameters including CH_4, pH and NH_3-N

The data for treatment effect on CH_4, pH, and NH_3-N are summarized in tables 3.3-3.5. The treatments significantly reduced CH_4 compared to the control. At 6 h of incubation, CH_4 decreased to 85, 94, and 99% of the control value by NS, NSP, and NSPB combination treatments, respectively, in the HF diet (tables 3.3), 84, 91, and 98% in the MF diet (table 3.4), and 84, 90, and 99% in the LF diet (table 3.5). The NS treatment decreased CH_4 by roughly 85% regardless of the diet at 6 h and roughly 93% in the HF and MF diets and 77% in the LF diet at 24 h of incubation (tables 3.3-3.5). The NSP treatment decreased CH_4 slightly more than NS; 95-98% inhibition of CH_4 compared to the control was observed for the NSP treatment (tables 3.3-3.5). The NSPB treatment completely inhibited CH_4, and values were reduced by 98% or more of the control value regardless of the diet and hour (tables 3.3-3.5).

Compared to the control, pH was significantly elevated by the treatments regardless of the diet and hour (tables 3.3-3.5). NH_3-N significantly increased compared to the control at 24 h.

Effect of combined treatment with $NaNO_3$, $NaSO_4$, 3NPA, and BCM on *in-vitro* SCFA

The data for treatment effect on SCFAs are summarized in tables 3.6-3.8. At 6 h, the NSPB treatment significantly decreased total SCFA in the HF diet (tables 3.6) and tended to decrease it in the MF diet (tables 3.7), compared to the control. There were no other significant changes on total SCFA compared to the control. However, the NSPB treatment in the HF and MF diet decreased acetate molar proportion at 24 h compared to the control (tables 3.6 & 3.7). On the other hand, propionate increased significantly compared to the control in the HF and MF diets regardless of the treatment combination (tables 3.6 & 3.7). In the LF diet, propionate tended to increase with the NSP and NSPB treatments compared to the control (table 3.8). These changes in acetate and propionate are reflected in the decreased acetate-to-propionate ratio (A:P) for the NSPB treatment in the HF and MF diets (tables 3.6 & 3.7).

On the other hand, butyrate molar proportion seems to have been decreased significantly by the NSP treatments in the MF and LF diets (tables 3.7 & 3.8). Additionally, butyrate was either unaffected in the HF and MF diets (tables 3.6 & 3.7) or significantly increased in the LF diet (table 3.8) by the NSPB treatment. Further, valerate increased significantly compared to the control in the HF and LF diets (tables 3.6 & 3.8) and tended to increase in the MF diet (table 3.7). The effect of combined treatments on the isoacids was largely inconsistent and insignificant (tables 3.6-3.8).

3.5 Discussion

The anti-methanogenic effects of combined H_2 sinks

In the present study, the NS and NSP treatments contained 2 and 3 potential H_2 sinks, respectively, namely NO_3^-, SO_4^{2-}, and 3NPA, to investigate the effect of concurrent supplementation of multiple H_2 sinks on ruminal methanogenesis. There was a significant

decrease in CH_4 by the NS and NSP treatments compared to the control (figure 3.2). However, the difference in CH_4 between these treatments was not significant, except in the LF diet at 24 h (table 3.5; figure 3.2). Nevertheless, there was a numerical decrease between the NS and NSP treatments in all diets and hours. It is inconclusive whether the addition of 3NPA to the NS treatment suppressed CH_4 even more than the NS treatment by itself. Inclusion of $NaNO_3$, $NaSO_4$, and 3NPA as individual treatments in the batch runs would have allowed for comparative analyses to clarify the contribution of individual compounds to the combination treatments.

With regards to the combination of NO_3^- and SO_4^{2-}, Van Zijderveld et al. (2010) showed that there was no additive effect of NO_3^- and SO_4^{2-} when included at 26 g/kg of diet while individual inclusion of these two compounds significantly reduced CH_4 *in vivo*. We observed a similar CH_4 inhibition by the 28g/kg inclusion of $NaNO_3$ in Experiment 1 (page 1). The suppression of CH_4 by the NS treatment in the present study may largely be due to the contribution of $NaNO_3$ because NO_3^- is considerably more effective as an anti-methanogenic agent than SO_4^{2-}. The dissimilatory nitrate reduction to ammonia (DNRA) is thermodynamically more favorable than the reduction of sulfate to hydrogen sulfide, i.e. $-\Delta G^o$ = 599.6 kJ/mol of DNRA (Thauer et al., 1977) *vs.* $-\Delta G^o$ = 234 kJ/mol of sulfate reduction to hydrogen sulfide (Ungerfeld & Kohn, 2006).

The 12 g/kg DM $NaSO_4$ inclusion level was most likely insufficient to pose a significant anti-methanogenic effect. In the present study, the 12 g/kg DM $NaSO_4$ inclusion level supplied 2.8 mM of SO_4^{2-}. This concentration falls below the 20 mM SO_4^{2-} inclusion level, at which SO_4^{2-} started to inhibit methanogenesis in a study by Ohashi et al. (1996). Therefore, it is most likely

that 28 g/kg DM $NaNO_3$ inclusion in the NS treatment was the dominant contributor to its anti-methanogenic effect observed in the present study.

The numerical decline of CH_4 in the NSP treatment compared to the NS may indicate additivity of these three compounds as anti-methanogenic agents. In fact, the NS and NSP treatments exerted similar effects on fermentation parameters other than CH_4 overall, with several numerical but statistically insignificant differences.

The effect of multiple H_2 sinks and a direct CH_4 inhibitor on ruminal fermentation

Another objective of the present study was to investigate the effects of concurrent supplementations of multiple H_2 sinks and a CH_4 analogue on methanogenesis in the NSPB treatment, representing inhibition of CH_4 simultaneously via thermodynamic and kinetic means. As expected, the NSPB treatment reduced CH_4 to a negligible level, hence achieving complete inhibition of CH_4 (figure 3.2). Compared to the NS treatment, the BCM inclusion exerted its inhibitory effect on CH_4 in the NSPB treatment, which was sustained for 24 h (figure 3.2).

Inclusion of BCM alone has been reported to achieve nearly complete inhibition of methanogenesis *in vitro* and *in vivo* (Trei & Olson, 1969; Trei et al., 1970, Sawyer et al., 1974). Similarly, we observed complete inhibition of methanogenesis at the 0.075, 0.15, & 0.30 g/kg DM inclusion levels of BCM in Experiment 2 (page 1). However, concurrent inclusion of halogenated methane analogue such as BCM and alternative H_2 sinks in the forms of $NaNO_3$, $NaSO_4$, and 3NPA has not received extensive investigation. The result of our present study indicates that the availability of alternative H_2 sinks does not interfere with the anti-methanogenic activity of BCM. This may be due to the difference between kinetic and thermodynamic manners of CH_4 inhibition. BCM kinetically inhibits methanogenesis by

alkylation of reduced vitamin B_{12}, which is then unable to participate in the final methyl transfer step of methanogenesis (Wood et al., 1968).

Because of the functional role of CH_4 as a primary H_2 sink, the inhibition of CH_4 by BCM results in the accumulation of H_2, which has been reported *in vitro* (Trei et al., 1970; Goel et al., 2009) and *in vivo* (Abecia et al., 2012; Mitsumori et al., 2012). This observation of elevated H_2 led to the concomitant use of H_2 sinks in order to examine the redirection of H_2 flow from methanogenesis to other H_2 sinks such as ammonia (NH_3) and SCFAs. Compared to the control in the present study, NH_3-N increased in the NS, NSP, and NSPB treatments. However, NH_3-N in the NSPB treatment did not differ from that in the NS and NSP treatments significantly; it may therefore be speculated that the elevation of NH_3-N was largely due to the $NaNO_3$ inclusion and that H_2 spared from methanogenesis, particularly with the CH_4 analogue, is not assimilated into NH_3.

There was no significant difference in total SCFA among all the treatments including the control at the end of incubation (tables 3.6-3.8). However, in the NSPB treatment, acetate decreased significantly compared to the rest of treatments in the HF and MF diets and tended to decrease compared to the NS and NSP treatments in the LF diet (figure 3.5). Among the treatments, NO_3^- has been reported to increase acetate by bypassing butyrate production (Farra & Satter, 1971; Anderson & Rasmussen, 1998; Sar et al., 2005; Latham et al., 2016). In the NS and NSP treatments, the increased acetate and decreased butyrate are consistent with the above pattern of NO_3^- effect on fermentation profile.

Yet, the effect of NO_3^- does not appear to be the sole contributor of the altered fermentation profile in the NS and NSP treatments as evidenced in the increased propionate on all treatments compared to the control (figure 3.6), which is inconsistent with the other studies

using NO_3^- (Latham et al., 2016). In the NSP treatment, 3NPA may have boosted the propionate production as reported by Anderson and Rasmussen (1998). The initially proposed metabolism of 3NPA by Majak and Cheng (1981) involves the formation of propionate via cleavage of the nitro-moiety from the compound, releasing NO_2^- as a result. However, Anderson et al. (1993) countered that the nitro-moiety may be reduced to an amine group without the detachment from the rest of the compound, forming *β*-alanine, which is further metabolized in the rumen. Although its metabolic fate is not extensively studied, *β*-alanine is a key part of Coenzyme A (CoA) biosynthesis as the constituent, pantothenate (vitamin B_5), contains *β*-alanine (Jin et al., 2018). This closely ties into NH_3 assimilation, which stimulates valine biosynthesis providing substrate for pantothenate synthesis (Jin et al., 2018). Since the NSP treatment contained both NO_3^- and 3NPA, substrates for NH_3 and *β*-alanine, respectively, it is most likely that the CoA biosynthesis was enhanced. Yet, it remains unclear whether the observed increase in propionate was a product of 3NPA metabolism theorized by Majak and Cheng (1981) or Anderson et al. (1993).

As for the NSPB treatment in the present study, reported shifts in fermentation pattern by BCM addition is consistent with other studies showing depressed acetate and elevated propionate (McCrabb et al., 1997; Denman et al., 2007; Goel et al., 2009), as well as butyrate in some cases (Goel et al., 2009; Sirohi & Goel, 2013). Interestingly in the present study, valerate was also increased in the NSPB treatment compared to the control and other treatments (figure 3.7). Formation of valerate occurs via the condensation of acetyl-CoA and propionyl-CoA (Van Soest, 1994; Yoshikawa et al., 2018), whose formation from acetate and propionate, respectively, may have been promoted by the abundance of CoA assuming that the contribution of NO_3^- and 3NPA on the pantothenate biosynthesis was significant (Yoshikawa et al., 2018).

Compared to the NS and NSP treatments, the inclusion of BCM in the NSPB treatment increased butyrate up to the control level (figure 3.7) while the A:P in the NSPB treatment was the lowest numerically among all of the treatments (figure 3.8). The decreased butyrate in the NS and NSP treatments indicates the formation of H_2 from protons via ferredoxin-dependent and ferredoxin- and NAD^+-dependent electron-bifurcating [FeFe]-hydrogenases (Zheng et al., 2014); H_2 is then utilized by the reduction of NO_3^- and SO_4^{2-}, and NADH is then unavailable for the conversion of acetoacetyl-CoA to butyryl-CoA in the butyrate pathway from acetyl-CoA (Macfarlane & Macfarlane, 2003). It may therefore be speculated that, since the inclusion of BCM in the NSPB treatment spared H_2 from methanogenesis, the availability of additional NADH was sufficient for butyrate production.

Conclusion

In conclusion, the combination of $NaNO_3$, $NaSO_4$, 3NPA, and BCM caused a significant shift in CH_4 and SCFAs predominantly mimicking the fermentation shift caused by individual BCM addition while pH and NH_3-N were similar to those observed in individual $NaNO_3$ addition. The results of the present study point to two major discoveries: first, that the highly efficacious, kinetic inhibition of CH_4 by BCM was not interfered by the thermodynamic inhibition by the alternative H_2 sinks used in the study; second, that shift in other fermentation parameters such as NH_3-N and SCFAs may be additive. Particularly, the fermentation pattern in the NSPB treatment indicates a favorable shift in ruminal fermentation with respect to animal performance and production as NH_3, propionate, butyrate, and valerate promotes microbial protein synthesis, host growth, postnatal epithelial cell proliferation, and fiber digestibility (Jin et al., 2018; Xue et al., 2020; Sakata & Tamate, 1978; Andries et al., 1987). Our prospective

research interest is to elucidate the treatment effects on microbial populations in these samples thereby obtaining additional insights on the phenomenon observed in this study.

3.6 Tables

Table 3.1. Formulation of three basal diets (high, medium, and low forage) in a 500 g batch used for the experiments

Ingredients	Basal diets		
	High forage (F:C=70:30)	Medium forage (50:50)	Low forage (30:70,)
Alfalfa pellets (F), *g*	350	250	150
Concentrate mix[1] (C), *g*	150	250	350
Ground corn, *g*	119.8	204.4	288.7
Soybean meal, *g*	25.2	40.6	56.3
Vitamin & mineral premix[2], *g*	5.0	5.0	5.0
Zn, *g*	0.06	0.06	0.06
Cu, *mg*	16.9	16.9	16.9
Co, *mg*	0.13	0.13	0.13
I, *mg*	1.30	1.30	1.30
Fe, *mg*	6.50	6.50	6.50
Mn, *mg*	39.0	39.0	39.0
Se, *mg*	0.39	0.39	0.39
Vitamin A, *IU*	8267	8267	8267
Vitamin D_3, *IU*	1102	1102	1102
Vitamin E, *IU*	5.51	5.51	5.51
Total, *g*	500	500	500

[1]Concentrate mix included ground corn, soybean meal, and vitamin and mineral mix.
[2]Vitamin and mineral mix included 1.2% Zn, 3,380 ppm Cu, 26 ppm Co, 260 ppm I, 1,300 ppm Fe, 7,800 ppm Mn, 78 ppm Se, 750,000 IU/lb vitamin A, 100,000 IU/lb vitamin D_3, 5,000 IU/lb vitamin E.

Table 3.2. Chemical composition of three basal diets (high, medium, and low forage) on a dry matter basis (DM)

Chemical composition[1]	Basal diets		
	High Forage (F:C=70:30)	Medium Forage (50:50)	Low Forage (30:70)
Dry matter, *%*	88.8	88.4	88.0
Acid detergent fiber, *%*	27.1	20.7	14.2
Neutral detergent fiber, *%*	35.9	28.8	21.7
Crude protein[2], *%*	18.0	18.1	18.2
Metabolizable energy, *Mcal/kg DM*	2.28	2.35	2.42
Net energy, *Mcal/kg DM*	1.72	1.93	2.15
Ether extract, *%*	3.16	3.46	3.75
Ca, *%*	1.2	0.88	0.57
P, *%*	0.34	0.36	0.38
Mg, *%*	0.28	0.25	0.21
K, *%*	2.10	1.80	1.40
Na, *‰*	0.86	0.68	0.51
Cl, *%*	0.54	0.42	0.29
S, *%*	0.25	0.23	0.20

[1]Chemical composition of each basal diet was calculated from nutrient values obtained in NRC (2001) for alfalfa hay (International Feed #: 1-00-023), ground corn (4-02-854), and soybean meal (5-20-638) unless otherwise stated.
[2]Crude protein contents of ground corn and soybean meal were 16 and 48% DM, respectively.

Table 3.3. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
CH_4, *mM*	1.98[a]	0.30[b]	0.12[bc]	0.02[c]	0.06
pH	5.84[a]	6.12[b]	6.11[b]	6.06[b]	0.03
NH_3-N, *mg/dL*	13.76[a]	25.04[b]	20.28[ab]	19.88[ab]	3.48
24 hours					
CH_4, *mM*	4.56[a]	0.29[b]	0.22[b]	0.02[b]	0.10
pH	5.42[a]	5.72[b]	5.66[c]	5.65[c]	0.01
NH_3-N, *mg/dL*	15.01[a]	22.92[b]	22.76[b]	25.11[b]	3.52

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

Table 3.4. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6, 12, and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
CH_4, *mM*	1.98[a]	0.32[b]	0.17[bc]	0.03[c]	0.06
pH	5.84[a]	6.11[b]	6.10[b]	6.09[b]	0.02
NH_3-N, *mg/dL*	16.22[a]	24.94[b]	21.88[bc]	19.02[ac]	3.12
24 hours					
CH_4, *mM*	4.75[a]	0.34[b]	0.11[b]	0.02[b]	0.12
pH	5.33[a]	5.59[b]	5.59[b]	5.56[b]	0.01
NH_3-N, *mg/dL*	13.54[a]	23.02[b]	19.99[b]	22.97[b]	3.14

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

Table 3.5. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on methane (CH_4), pH, and ammonia-N (NH_3-N) in mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6 and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
CH_4, *mM*	1.94[a]	0.31[b]	0.19[b]	0.02[b]	0.14
pH	5.90[a]	6.14[b]	6.07[b]	6.13[b]	0.02
NH_3-N, *mg/dL*	19.02	24.67	21.52	25.27	3.51
24 hours					
CH_4, *mM*	4.39[a]	1.00[b]	0.12[c]	0.02[c]	0.17
pH	5.29[a]	5.50[b]	5.53[b]	5.47[c]	0.01
NH_3-N, *mg/dL*	11.86[a]	21.00[b]	20.79[b]	20.64[b]	1.86

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

Table 3.6. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a high forage diet (70:30 forage to concentrate[1] ratio) and incubated at 39°C for 6 and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
Total SCFA, *mM*	99.44[a]	100.51[a]	96.49[a]	68.04[b]	7.69
Individual SCFA	——————————*mol %*——————————				
Acetate (A)	64.68	67.78	66.58	62.72	2.04
Propionate (P)	21.54[de]	19.54[d]	20.39[de]	23.86[e]	1.12
Butyrate	10.18	9.32	9.08	9.14	0.62
Isobutyrate	1.63	0.89	1.38	2.10	0.51
Valerate	0.99	1.32	1.29	1.34	0.18
Isovalerate	0.98[ac]	1.13[ab]	1.29[b]	0.84[c]	0.09
A:P Ratio	3.01	3.47	3.31	2.71	0.22
24 hours					
Total SCFA, *mM*	120.92	120.90	111.00	114.17	6.34
Individual SCFA	——————————*mol %*——————————				
Acetate (A)	60.09[a]	59.56[a]	56.34[a]	53.02[b]	1.68
Propionate (P)	21.33[a]	24.40[b]	24.66[b]	26.03[b]	0.61
Butyrate	15.58[ab]	12.54[a]	14.32[a]	17.37[b]	0.96
Isobutyrate	0.62[a]	1.04[ab]	1.88[b]	0.69[a]	0.33
Valerate	1.14[a]	1.58[ab]	1.76[bc]	1.94[c]	0.13
Isovalerate	0.97	0.98	1.04	0.95	0.08
A:P Ratio	2.83[a]	2.44[b]	2.29[bc]	2.05[c]	0.12

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

Table 3.7. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a medium forage diet (50:50 forage to concentrate[1] ratio) and incubated at 39°C for 6 and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
Total SCFA, *mM*	92.14[d]	96.59[d]	84.20[de]	66.69[e]	7.20
Individual SCFA			*mol %*		
Acetate (A)	63.96	68.06	66.48	62.72	2.02
Propionate (P)	20.42	19.05	20.70	22.25	1.13
Butyrate	12.04	9.43	9.64	10.82	0.99
Isobutyrate	1.35	0.99	1.09	1.46	0.38
Valerate	1.23	1.32	1.22	1.40	0.18
Isovalerate	1.00	1.14	1.38	1.36	0.19
A:P Ratio	3.14	3.57	3.30	2.86	0.24
24 hours					
Total SCFA, *mM*	126.55	123.28	113.77	118.15	6.44
Individual SCFA			*mol %*		
Acetate (A)	55.93[a]	55.59[a]	53.61[ab]	50.05[b]	1.37
Propionate (P)	20.56[a]	25.86[b]	26.29[b]	26.00[b]	0.49
Butyrate	19.83[a]	15.09[b]	16.14[b]	19.75[a]	1.00
Isobutyrate	1.06	0.82	1.47	1.16	0.37
Valerate	1.53[d]	1.68[de]	1.68[de]	2.02[e]	0.12
Isovalerate	1.09	0.96	0.81	1.02	0.09
A:P Ratio	2.72[a]	2.15[b]	2.05[bc]	1.93[c]	0.08

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

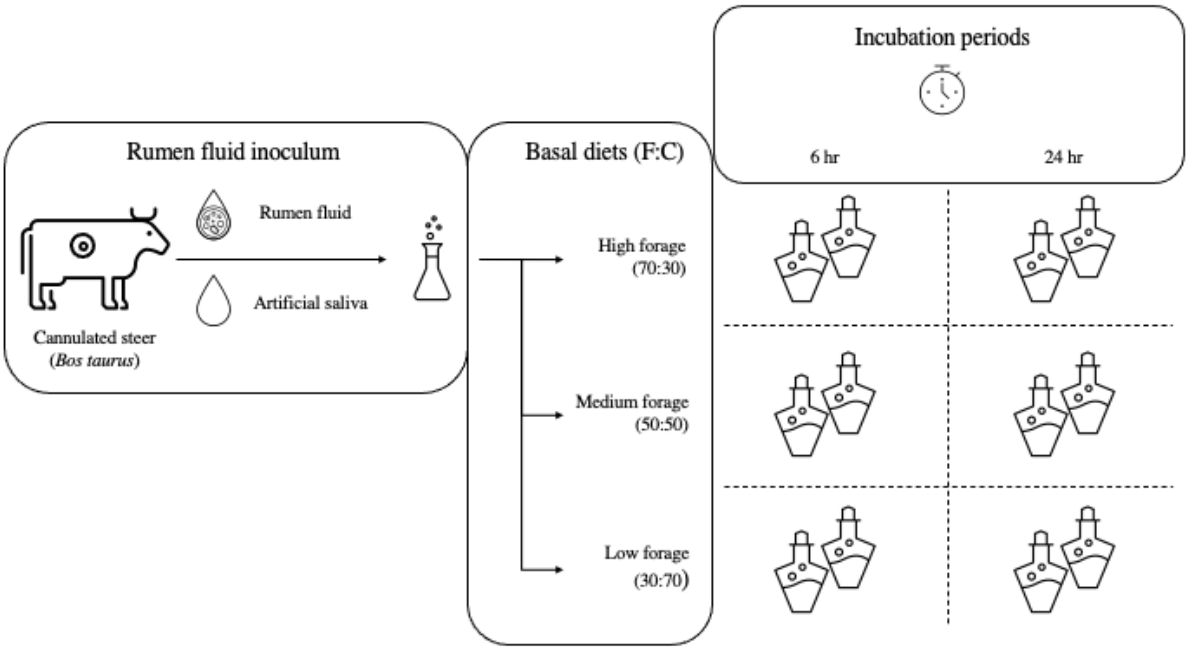
Table 3.8. Effect of combining sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on short-chain fatty acids (SCFA) from mixed cultures of rumen microbes fed a low forage diet (30:70 forage to concentrate[1] ratio) and incubated at 39°C for 6 and 24 hours.

	Treatments				
	C[2]	NS[3]	NSP[4]	NSPB[5]	SEM[6]
6 hours					
Total SCFA, *mM*	86.37	87.27	92.42	74.68	6.02
Individual SCFA	——————————*mol %*——————————				
Acetate (A)	62.93[a]	66.82[ab]	70.24[b]	67.29[ab]	1.43
Propionate (P)	19.44	19.40	17.64	19.91	0.87
Butyrate	13.46[a]	10.09[b]	8.83[b]	9.31[b]	0.74
Isobutyrate	1.92	1.14	1.22	1.15	0.33
Valerate	1.24	1.34	1.01	1.15	0.13
Isovalerate	1.00	1.20	1.05	1.18	0.11
A:P Ratio	3.26	3.49	4.00	3.40	0.21
24 hours					
Total SCFA, *mM*	130.70	125.40	110.97	120.58	7.25
Individual SCFA	——————————*mol %*——————————				
Acetate (A)	50.24[de]	52.13[d]	51.88[d]	42.10[e]	3.03
Propionate (P)	22.82[d]	25.54[de]	26.29[e]	26.78[e]	1.27
Butyrate	23.62[ac]	18.92[ab]	18.72[b]	26.99[c]	1.77
Isobutyrate	0.73	0.74	0.67	0.87	0.22
Valerate	1.45[a]	1.65[a]	1.67[a]	2.12[b]	0.14
Isovalerate	1.14	1.01	0.94	1.14	0.12
A:P Ratio	2.28	2.04	1.97	1.61	0.20

[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Concentrate consisted of corn and soybean meal.
[2]C = Control, no additive
[3]NS = Sodium nitrate + Sodium sulfate (28 and 12 g/kg DM, respectively)
[4]NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM)
[5]NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[6]Standard error of the mean

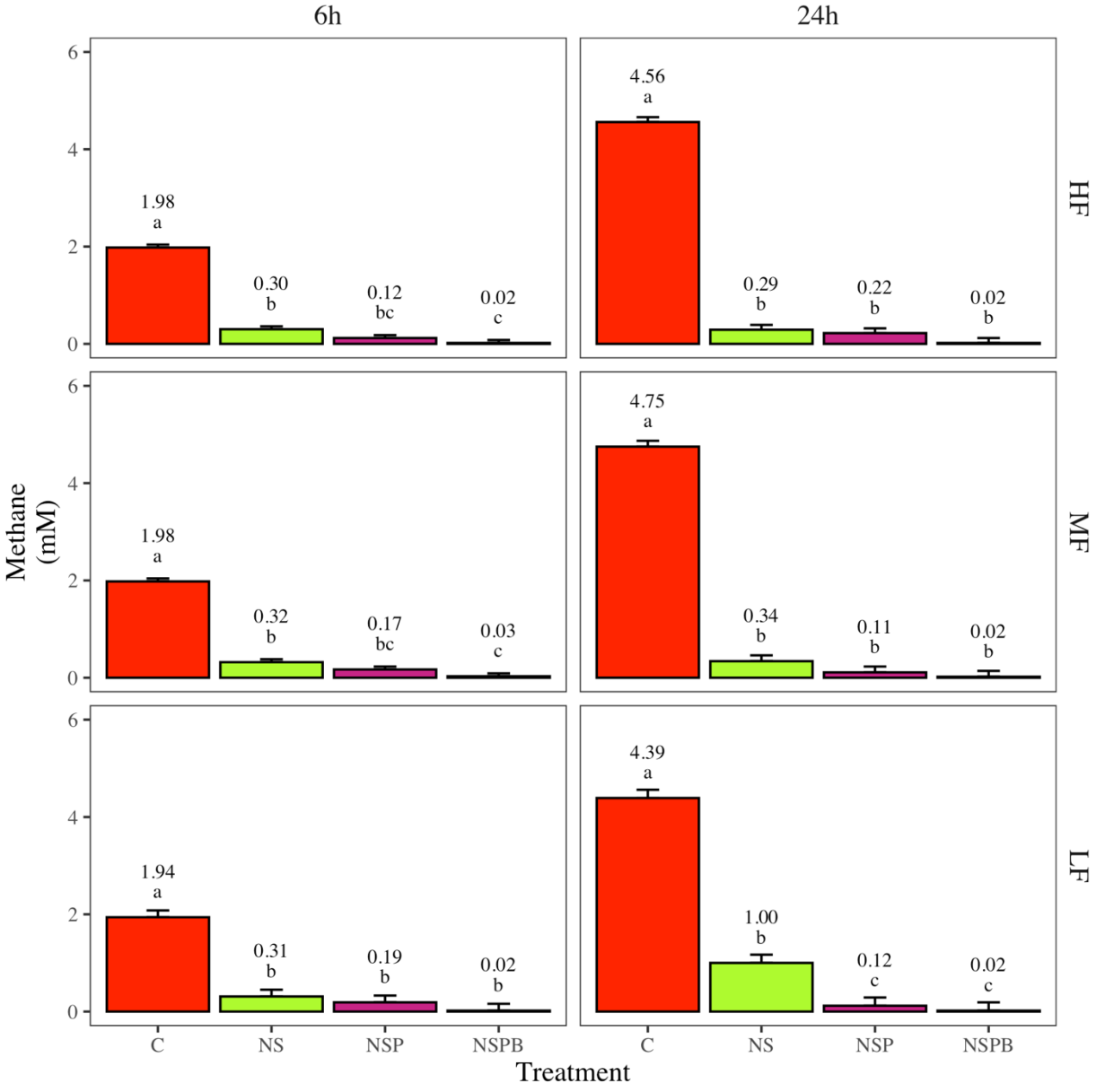
3.7 Figures

Figure 3.1. Simplified experimental design of *in-vitro* fermentation per treatment with combinations[1] of sodium nitrate, sodium sulfate, 3-nitro-1-propionate, and bromochloromethane in a batch run.



[1]Treatment combinations included: Control = No additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

Figure 3.2. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on methane in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.

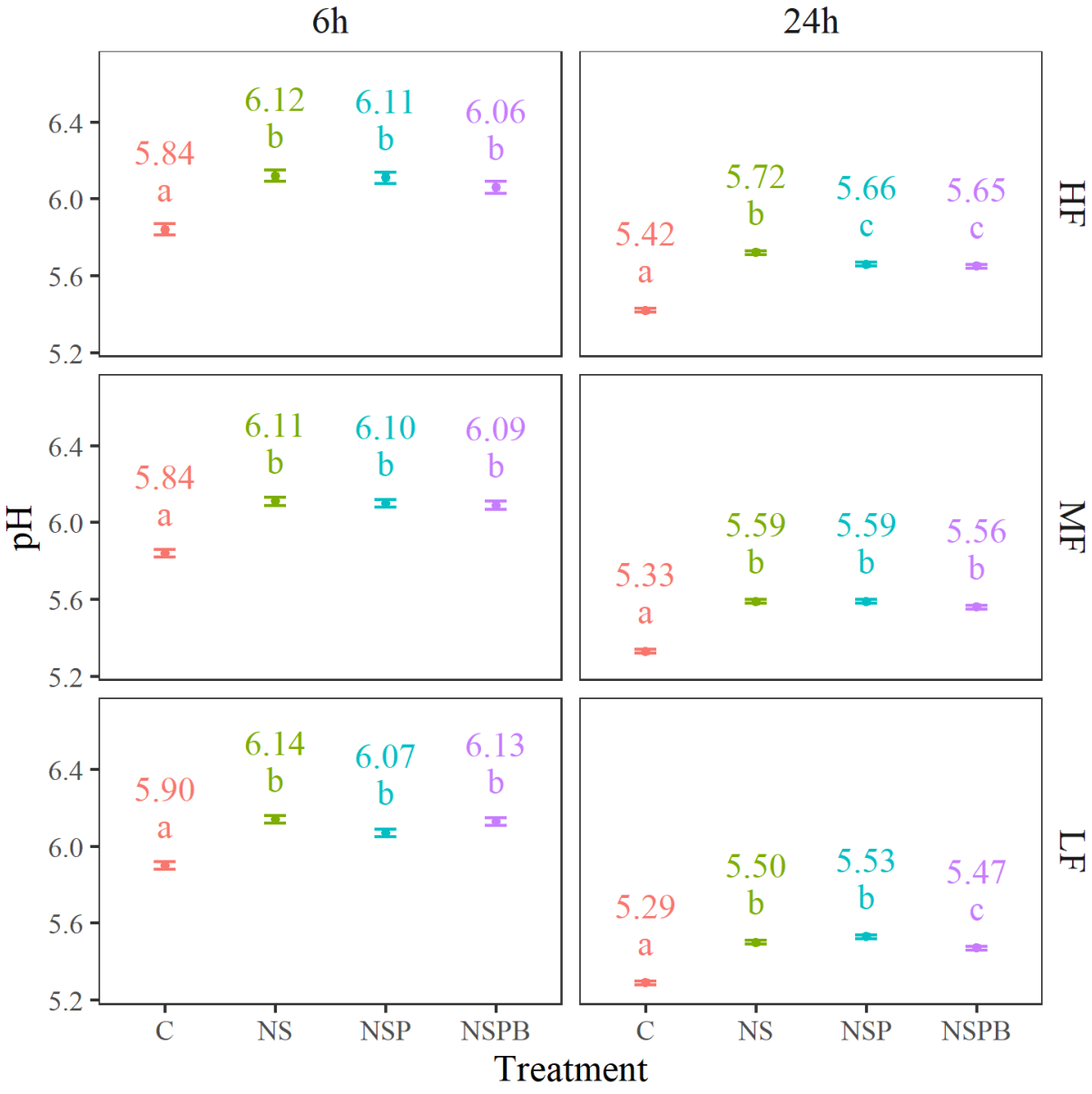


[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

[a-c]different superscripts within the hour and diet significantly differ ($p \leq 0.05$)

Figure 3.3. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on pH in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.

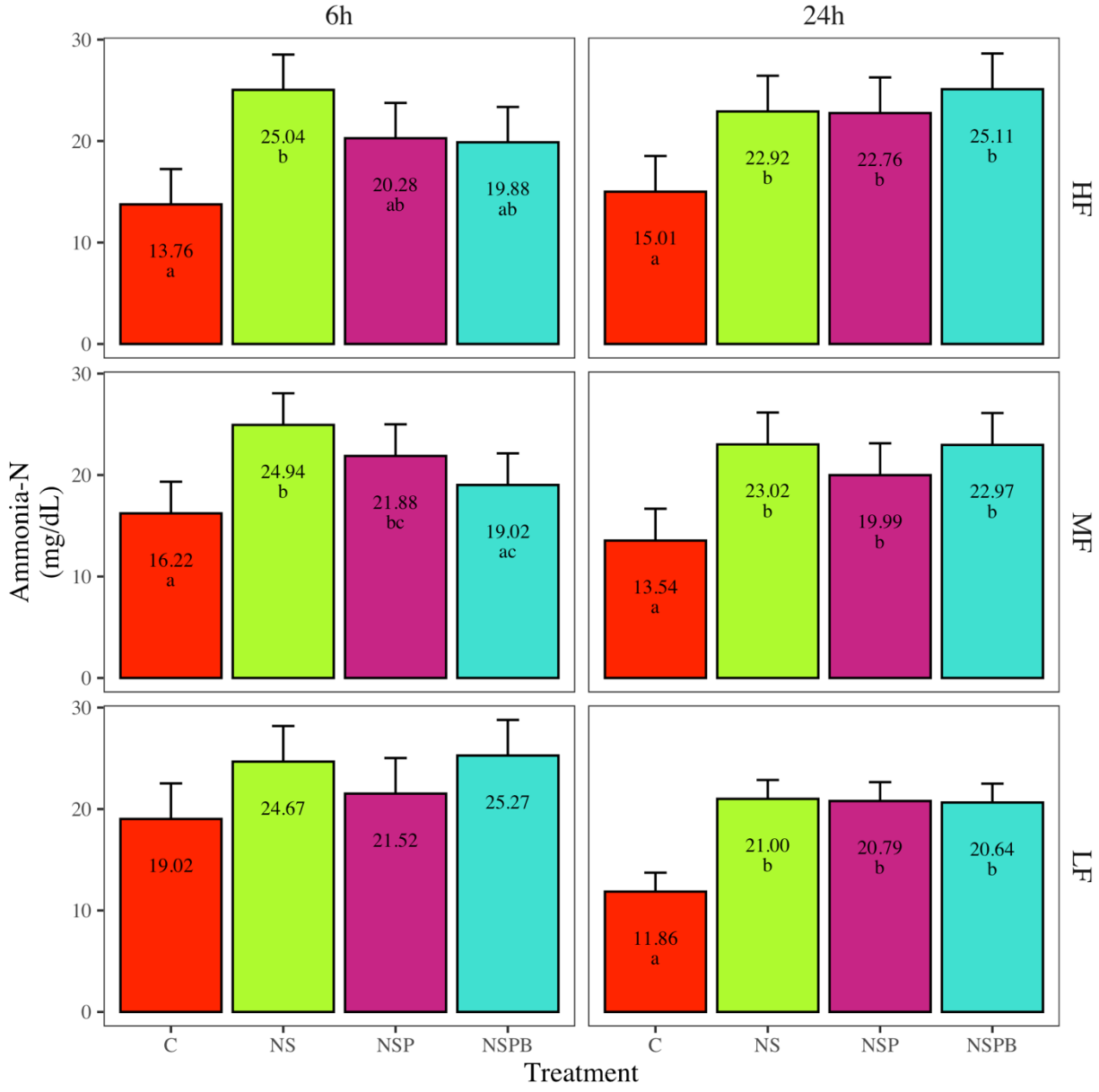


[a-c]different superscripts within a row differ ($p \leq 0.05$)

[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.4. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on ammonia-N in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.

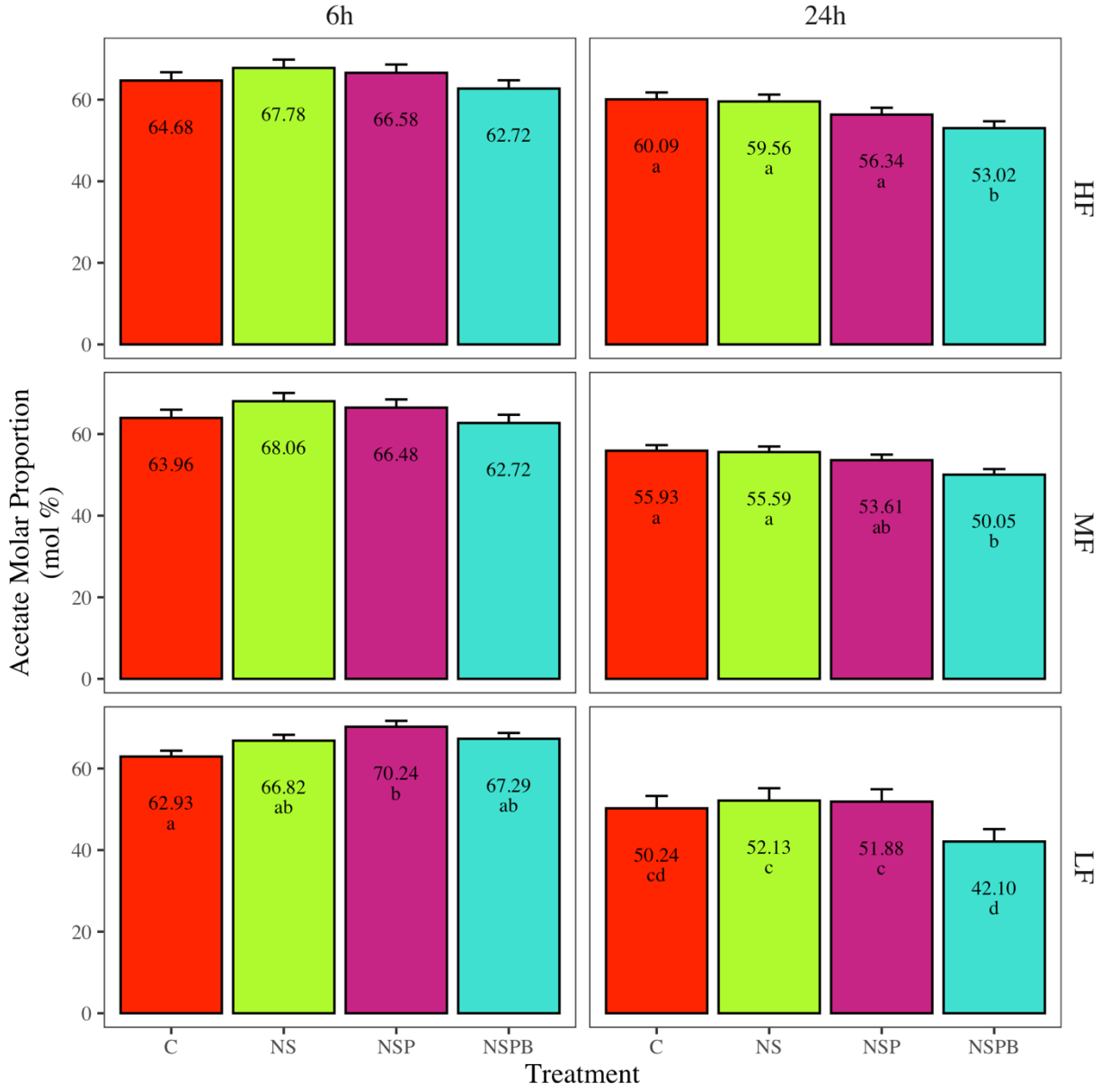


[a-c]different superscripts within a row differ ($p \leq 0.05$)

[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

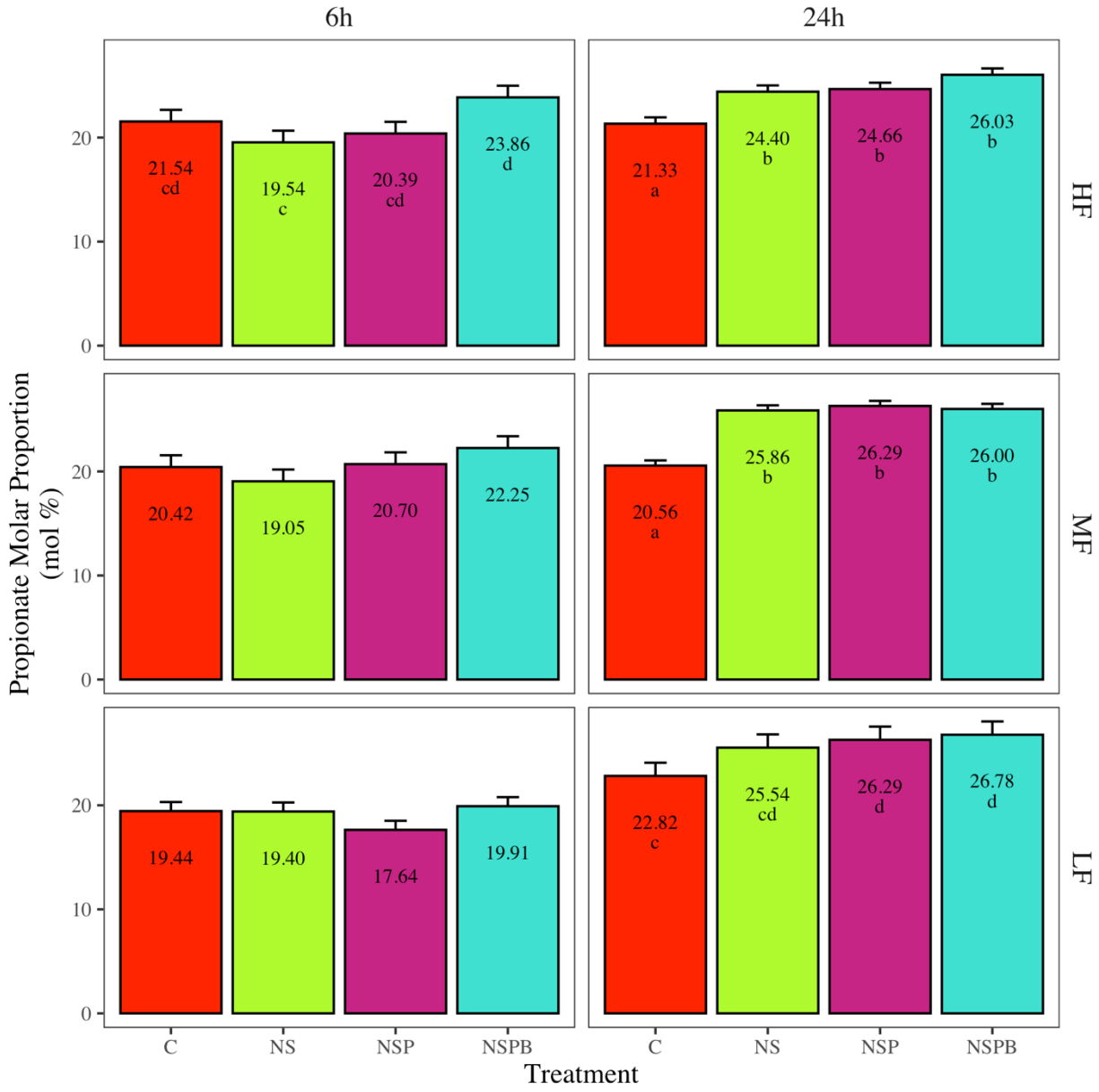
[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.5. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on acetate molar proportion in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.



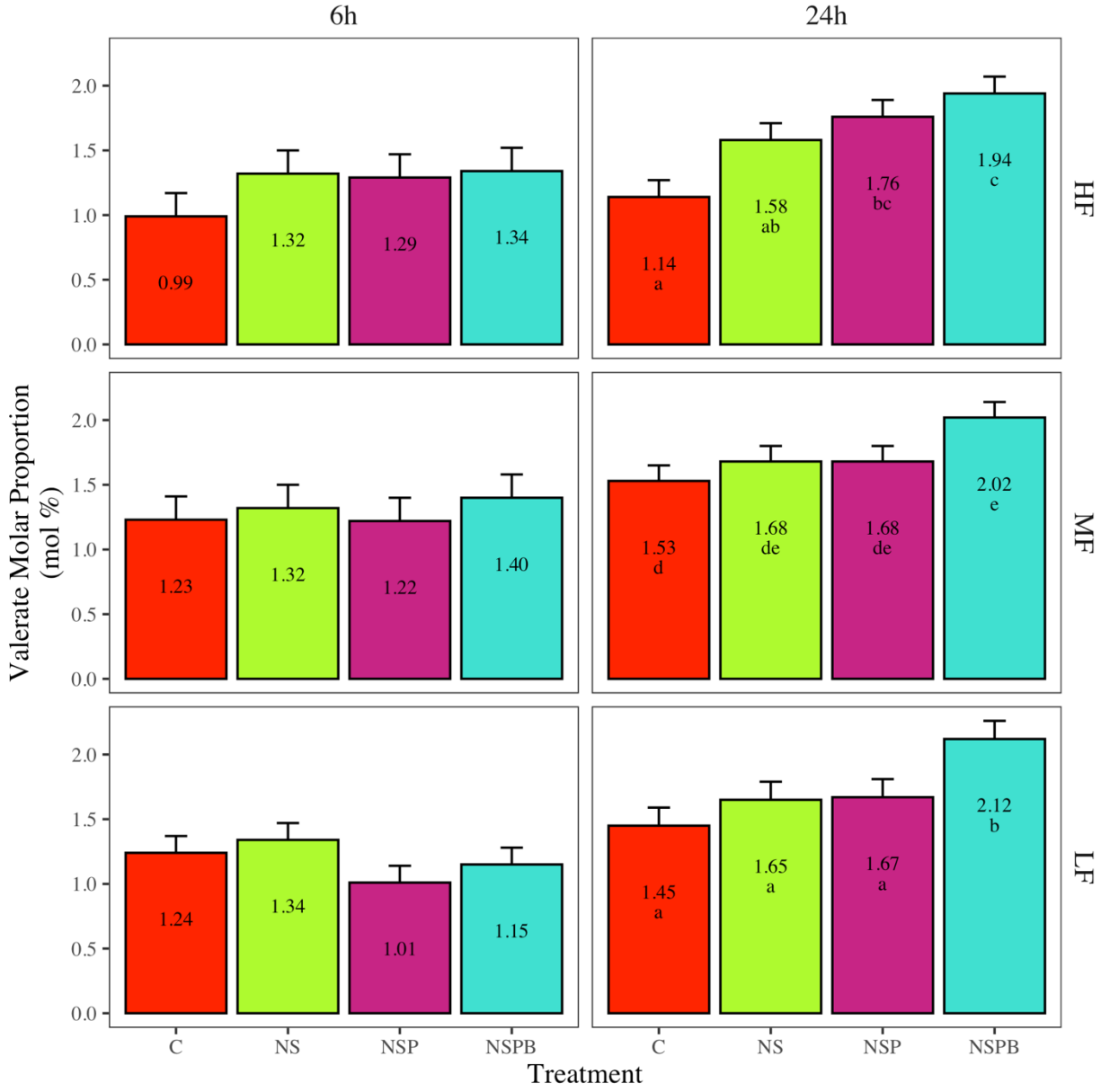
[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.6. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on propionate molar proportion in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.



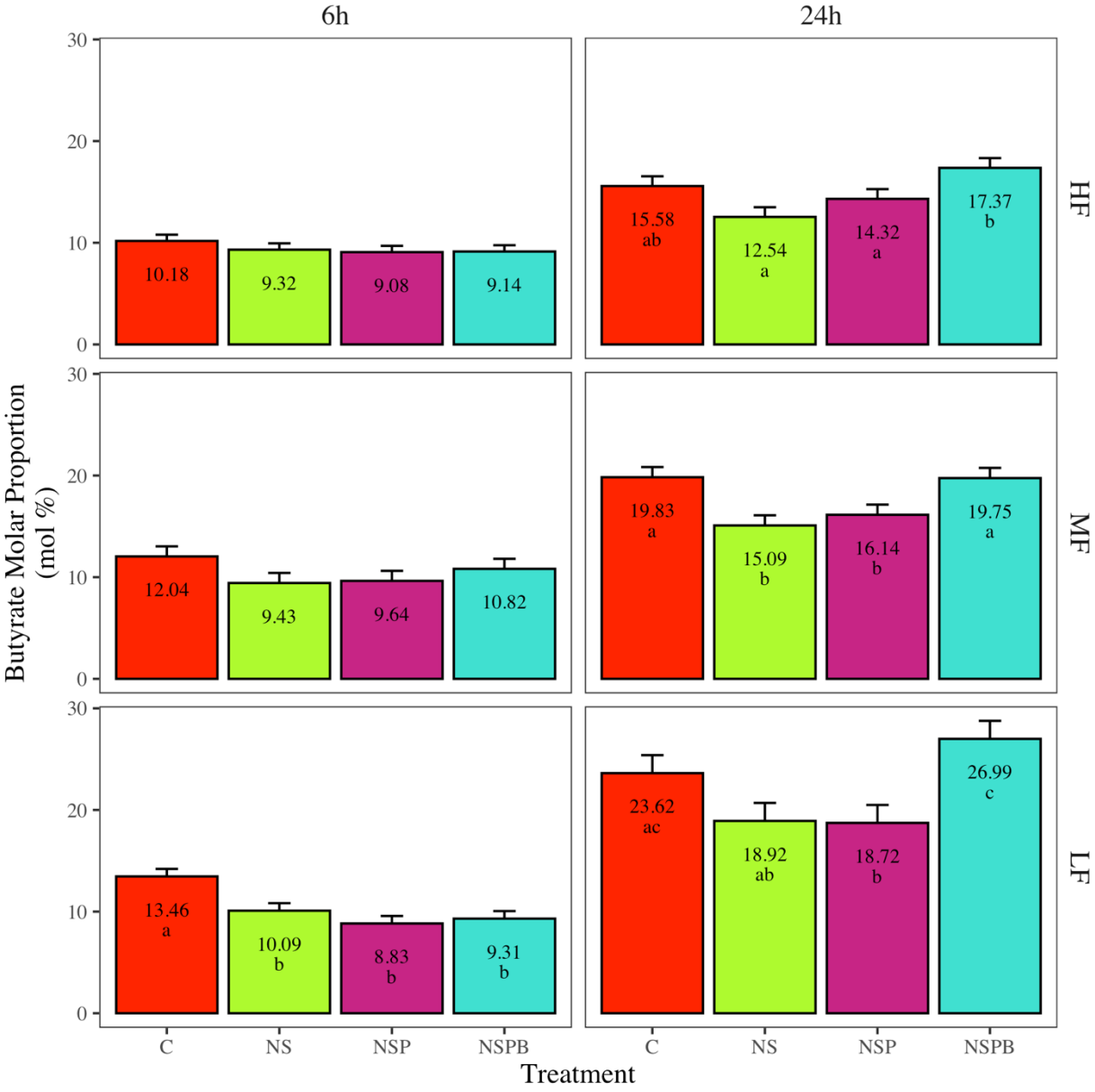
[a, b]different superscripts within a row differ ($p \leq 0.05$)
[c, d]different superscripts within a row differ ($p \leq 0.10$)
[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.7. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on valerate molar proportion in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.



[a-c]different superscripts within a row differ ($p \leq 0.05$)
[d, e]different superscripts within a row differ ($p \leq 0.10$)
[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)
[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.8. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on butyrate molar proportion in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.

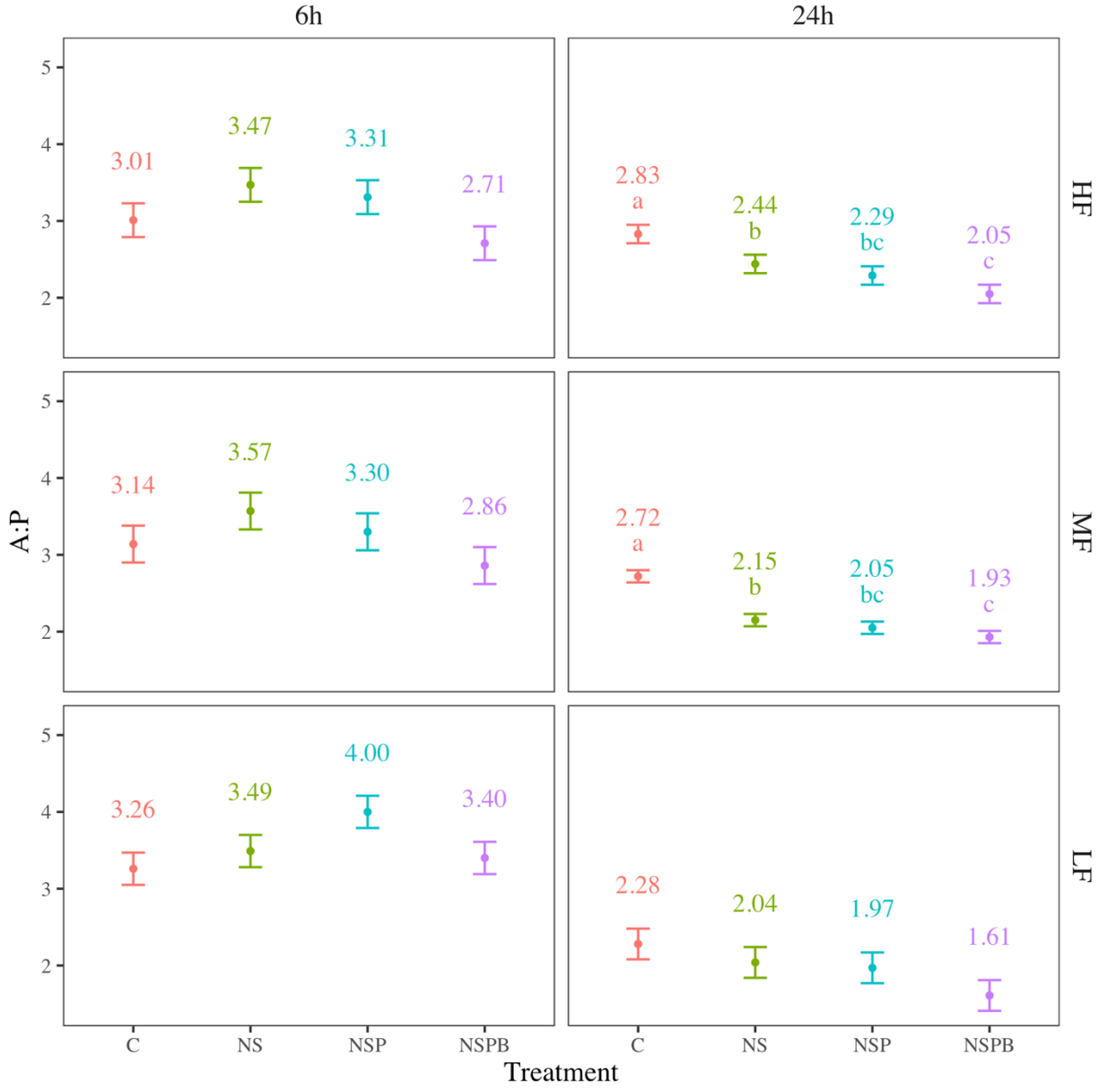


[a-c]different superscripts within a row differ ($p \leq 0.05$)

[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

Figure 3.9. Effect of combined treatments[1] with sodium nitrate (N), sodium sulfate (S), 3-nitro-1-propionate (P), and bromochloromethane (B) on acetate-to-propionate molar ratio in mixed cultures of rumen microbes fed diets[2] varying in forage-to-concentrate ratio and incubated at 39°C for 6 and 24 hours.



[a-c]different superscripts within a row differ ($p \leq 0.05$)

[1]Treatments: C = Control, no additive;
NS = $NaNO_3$ (28 g/kg DM) + Na_2SO_4 (12 g/kg DM);
NSP = NS + 3-nitro-1-propionate (2.0 g/kg DM);
NSPB = NSP + bromochloromethane (0.30 g/kg DM)

[2]Diets were prepared by mixing (DMB) alfalfa hay and concentrate mix (ground corn and soybean meal) respectively in 3 ratios: high forage (HF, 70:30), medium forage (MF, 50:50), and low forage (LF, 30:70).

REFERENCE

Abecia, L., Toral, P. G., Martín-García, A. I., Martínez, G., Tomkins, N. W., Molina-Alcaide, E., Newbold, C. J., & Yáñez-Ruiz, D. R. (2012). Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. *Journal of Dairy Science*, *95*(4), 2027–2036. https://doi.org/10.3168/jds.2011-4831

Andries, J. I., Buysse, F. X., De Brabander, D. L., & Cottyn, B. G. (1987). Isoacids in ruminant nutrition: Their role in ruminal and intermediary metabolism and possible influences on performances — A review. *Animal Feed Science and Technology*, *18*(3), 169–180. https://doi.org/10.1016/0377-8401(87)90069-1

Beecher, G. R., & Whitten, B. K. (1970). Ammonia determination: Reagent modification and interfering compounds. *Analytical Biochemistry*, *36*(1), 243–246. https://doi.org/10.1016/0003-2697(70)90356-8

Beerling, D., Berner, R. A., Mackenzie, F. T., Harfoot, M. B., & Pyle, J. A. (2009). Methane and the CH4 related greenhouse effect over the past 400 million years. *American Journal of Science*, *309*(2), 97–113. https://doi.org/10.2475/02.2009.01

Blaxter, K., & Clapperton, J. (1965). Prediction of the amount of methane produced by ruminants. *British Journal of Nutrition, 19*(1), 511-522. doi:10.1079/BJN19650046

Chalupa, W. (1977). Manipulating Rumen Fermentation, *Journal of Animal Science*, *45*(3), 585–599, https://doi.org/10.2527/jas1977.453585x

Correa, A. C., Trachsel, J., Allen, H. K., Corral-Luna, A., Gutierrez-Bañuelos, H., Ochoa-Garcia, P. A., Ruiz-Barrera, O., Hume, M. E., Callaway, T. R., Harvey, R. B., Beier, R. C., Anderson, R. C., & Nisbet, D. J. (2017). Effect of sole or combined administration of nitrate and 3-nitro-1-propionic acid on fermentation and Salmonella survivability in

alfalfa-fed rumen cultures in vitro. *Bioresource Technology*, *229*, 69–77. https://doi.org/10.1016/j.biortech.2017.01.012

Denman, S. E., Tomkins, N. W., & McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane, *FEMS Microbiology Ecology*, *62*(3), 313–322, https://doi.org/10.1111/j.1574-6941.2007.00394.x

Farra, P. A., and Satter, L. D. (1971). Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *Journal of Dairy Science*, *54*, 1018–1024. doi: 10.3168/jds.S0022-0302(71) 85965-9

Goel, G., Makkar, H. P., & Becker, K. (2009). Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. *The British Journal of Nutrition*, *101*(10), 1484–1492. https://doi.org/10.1017/S0007114508076198

Jin, D., Zhao, S. G., Zheng, N., Bu, D. P., Beckers, Y., & Wang, J. Q. (2018). Urea nitrogen induces changes in rumen microbial and host metabolic profiles in dairy cows. *Livestock Science*, *210*, 104–110. https://doi.org/10.1016/j.livsci.2018.02.011

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science*, *73*(8), 2483–2492. https://doi.org/10.2527/1995.7382483x

Kassambra, A. (2020). ggpubr: *'ggplot2' Based Publication Ready Plots. R package version 0.4.0, <https://CRAN.R-project.org/package=ggpubr>.*

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *The Proceedings of the Nutrition Society*, *62*(1), 67–72. https://doi.org/10.1079/PNS2002207

Majak, W., & Cheng, K. J. (1981). Identification of rumen bacteria that anaerobically degrade aliphatic nitrotoxins. *Canadian Journal of Microbiology*, *27*(7), 646–650. https://doi.org/10.1139/m81-099

McDougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *The Biochemical Journal*, *43*(1), 99–109.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., & McSweeney, C. S. (2012). Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *The British Journal of Nutrition*, *108*(3), 482–491. https://doi.org/10.1017/S0007114511005794

Morgavi, D. P., Forano, E., Martin, C., & Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal*, *4*(7), 1024–1036. https://doi.org/10.1017/S1751731110000546

Ohashi, Y., Ushida, K., Miyasaki, K., & Kojima, Y. (1996). Effect of initial sulfate level on electron partition between methanogenesis and sulfate reduction in the rumen. *Annales de Zootechnie*, *45*(Suppl. 1), 320–320. https://doi.org/10.1051/animres:19960660

Sakata, T., & Tamate, H. (1978). Rumen epithelial cell proliferation accelerated by rapid increase in intraruminal butyrate. *Journal of Dairy Science*, *61*(8), 1109–1113. https://doi.org/10.3168/jds.S0022-0302(78)83694-7

Sar, C., Mwenya, B., Pen, B., Takaura, K., Morikawa, R., Tsujimoto, A., et al. (2005). Effect of ruminal administration of *Escherichia coli* wild type or a genetically modified strain with enhanced high nitrite reductase activity on methane emission and nitrate toxicity in

nitrate-infused sheep. *British Journal of Nutrition*, *94*, 691–697. doi: 10.1079/BJN20051517

Sawyer, M. S., Hoover, W. H., & Sniffen, C. J. (1974). Effects of a ruminal methane inhibitor on growth and energy metabolism in the ovine. *Journal of Animal Science*, *38*(4), 908–914. https://doi.org/10.2527/jas1974.384908x

Sirohi, S., & Goel, N. (2013). Effect of various levels of bromochloromethane and fibre level on in vitro methane production and fermentation pattern. *The Indian Journal of Animal Science*, *83*(8), 798-801.

Thauer, R. K., Jungermann, K., & Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews*, *41*(1), 100–180. https://doi.org/10.1128/br.41.1.100-180.1977

Trei, J. E. & Olson, W. A. (1969). Effect of chlorine containing analogues of methane on rumen fermentation. *Journal of Animal Science*, *29*, 173-174.

Trei, J. E., Singh, Y. K., & Scott, G. C. (1970). Effect of methane inhibitors on rumen metabolism. *Journal of Animal Science*, *31*, 392.

Ungerfeld, E. M. (2020). Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, *11*, 589. https://doi.org/10.3389/fmicb.2020.00589

Ungerfeld, E., & Kohn, R. (2006). The Role of Thermodynamics in the Control of Ruminal Fermentation. In *Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress*. 55–85.

Van Soest, P. J. (1994). *Nutritional ecology of the ruminant*. Cornell University Press.

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

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, https://ggplot2.tidyverse.org.

Wolin, M. J. (1979). The Rumen Fermentation: A Model for Microbial Interactions in Anaerobic Ecosystems. In M. Alexander (Ed.), *Advances in Microbial Ecology*, *3*, 49–77. Springer US. https://doi.org/10.1007/978-1-4615-8279-3_2

Wood, J. M., Kennedy, D. S., & Wolfe, R. S. (1968). Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry*, *7*(5), 1707-1713. https://doi.org/10.1021/bi00845a013

Xue, M. Y., Sun, H. Z., Wu, X. H., Liu, J. X., & Guan, L. L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome*, *8*(1), 64. https://doi.org/10.1186/s40168-020-00819-8

Yoshikawa, S., Araoka, R., Kajihara, Y., Ito, T., Miyamoto, H., & Kodama, H. (2018). Valerate production by Megasphaera elsdenii isolated from pig feces. *Journal of Bioscience and Bioengineering*, *125*(5), 519–524. https://doi.org/10.1016/j.jbiosc.2017.12.016

Zheng, Y., Kahnt, J., Kwon, I. H., Mackie, R. I., & Thauer, R. K. (2014). Hydrogen Formation and Its Regulation in Ruminococcus albus: Involvement of an Electron-Bifurcating [FeFe]-Hydrogenase, of a Non-Electron-Bifurcating [FeFe]-Hydrogenase, and of a

Putative Hydrogen-Sensing [FeFe]-Hydrogenase. *Journal of Bacteriology*, *196*(22), 3840–3852. https://doi.org/10.1128/JB.02070-14

CONCLUSION

In conclusion, the anti-methanogenic effects of individual and concurrent supplementation of $NaNO_3$, $NaSO_4$, 3NPA, and BCM were various yet promising. The kinetic inhibition of methanogenesis by BCM was the most effective overall when included individually and in combination with $NaNO_3$, $NaSO_4$, and 3NPA. The efficacy of the anti-methanogenic effect of BCM was not interfered by the presence of alternative H_2 sinks. However, there was a clear difference in fermentation shift by the BCM inclusion depending on the supplementation of alternative H_2 sinks. Independently, BCM inclusion decreased acetate and increased propionate while liberating excess H_2. In the presence of alternative H_2 sinks, BCM inclusion also increased butyrate and valerate as well as propionate.

On the other hand, the thermodynamic inhibition by $NaNO_3$ was the second most effective overall and most effective among the alternative H_2 sinks used in the present study. The inclusion of $NaNO_3$ individually and in combination with other alternative H_2 sinks and BCM resulted in increased acetate, NH_3-N, and pH. This is expected because of the ruminal metabolism of NO_3^- to NH_3 as a H_2 sink, which serves as a buffer preventing low pH. Butyrate was decreased by $NaNO_3$ inclusion because the reduction of NO_3^- to NH_3 renders NADH unavailable for the butyrate pathway. However, under the complete inhibition of methanogenesis by BCM, NO_3^- served as one of the primary alternative H_2 sinks leading to NH_3 production, which may have promoted valine biosynthesis, while the excess H_2 was assimilated into the butyrate pathway.

The inclusion of 3NPA moderately decreased methanogenesis *in vitro* especially during an early stage of *in-vitro* fermentation. The mode of action of 3NPA has not been clearly elucidated; it is proposed that reduction of 3NPA to β-alanine acts as a H_2 sink. In combination

with enhanced valine biosynthesis via NH_3 production from $NaNO_3$ inclusion, *β*-alanine may have contributed to the biosynthesis of pantothenate (vitamin B_5) and subsequently of Coenzyme A (CoA). Therefore, we attribute the concurrent inclusion of $NaNO_3$ $NaSO_4$, 3NPA, and BCM to the increase of butyrate and valerate. Under the complete inhibition of methanogenesis by BCM, the availability of H_2 and CoA led to the assimilation of these molecules into production of propionate, butyrate, and valerate as internal H_2 sinks.

Despite the expected reduction of SO_4^{2-} to H_2S, the inclusion of $NaSO_4$ had no effect on the *in-vitro* methanogenesis and fermentation pattern due to the insufficient levels of inclusion in our present study. However, there may have been additive effects of $NaSO_4$ and other alternative H_2 sinks and BCM in the present study.

In the combination of kinetic and thermodynamic inhibition of methanogenesis, the observed shift in the *in-vitro* fermentation pattern may be appealing in terms of ruminant livestock production. In particular, propionate and valerate are gluconeogenic precursors in the liver of the animal, leading to extra supplies of energy utilized for growth, lactation, and pregnancy. Butyrate improves postnatal epithelial cell proliferation in the rumen. Ammonia is a microbially preferred source of non-protein nitrogen promoting microbial protein synthesis while also preventing low rumen pH and acidosis. For further investigation, we need to clarify the metabolic fates of alternative H_2 sinks thoroughly via direct measurements of their products and examine the effects of kinetic and thermodynamic inhibition of methanogenesis on microbial populations in the rumen content.