EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON BEEF CATTLE PRODUCTION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2017

To my wife and family

ACKNOWLEDGMENTS

I want to give my greatest thanks to our Lord and Savior, without him in my life, I would not have had any of the success I have enjoyed in my life.

My wife is the most important person in my life. It was because of her support, love, and unbelievable hard work, that I was able to complete my Doctor of philosophy degree. She has stood by my side through the often rewarding moments in a young scientist's life and held my hand when that can become seemingly unbearable. Thank you, Beeb, I love you from here all the way around to here.

Without the solid foundation provided by my parents, Sonny and Connie Henry, I would have never left Montgomery County, TX. Their love and support through my career as a professional student was, and always will be, more than I could ever ask for. I would also like to thank Katie Henry for her patience throughout this process as well. It would have been impossible to complete 3 experiments and plan a wedding at the same time without her. I need to thank my brother, little Sonny, for always being there for me. I wish to give a huge thank you to Ruth Henry (aka Mawmaw). She never gave up on me while I was away studying and she forgives me when I forget to call.

I hold an undying love in my heart for my family who are no longer with us. I want to thank Joseph F. Henry, Sr. for his unconditional love and support all of my life. To Mary Gribble, I give immense thanks for her love and laughter. I thank Jerry Caveness for teaching me how to "play opossum" and for letting me drive when I was 13. Howard Gribble was a man I will never forget and I will miss him dearly for all of my days.

I give great gratitude to Dr. Rafael C. Araujo and his employers, GRASP Ind. & Com, for providing all of the nitrate used during my PhD program. Furthermore, I wish to thank Rafael especially for his comments and valuable inputs to the design of the

experiments. Mr. Randy Davis and Quality Liquid Feeds deserves my thanks for donating the molasses for these experiments. More than that, I thank Randy for the amazing crawfish boil that he performed for Francine and myself as a wedding gift. C'est de la bonne merde.

I would like to extend my gratitude to my friend, Dr. Vitor Mercadante. Vitor was a great example to follow throughout my graduate career and the lessons I learned from him are invaluable. Pedro Fontes is also a fellow student whom I would like to give special recognition. It is easy to become content with the work you are doing, and Pedro's hunger and curiosity always kept me on my toes. A huge thanks goes out to Dr. Martin Ruiz-Moreno for all of the insightful discussions we have had over the years. I would also like to extend my gratitude to all of my friends and colleagues who have helped me over the years: Nicky Oosthuizen, Erick Santos, David Jaramillo, Lautaro Rostoll, Mariana Garcia, Tessa Schulmeister, Carla Sanford, Luara Canal, and many others.

I would like to thank the countless interns from all over the world who lent an extremely big hand in collecting the data for these experiments. In particular I would like to give a big "thank you" to Sebastian Mejia, Federico Podversich, Pedro Sueldo, and Maria Alejandra Gutierrez Beltrán.

I wish to thank the members of my committee: Drs. G. Clifford Lamb, Jose Dubeux, Adegbola Adesogan, and K. C. Jeong. Your patience with me and your guidance over the past years has been invaluable.

I have the utmost love and respect for Dr. Nicolas DiLorenzo. As a mentor and advisor, I can think of none better than him. Thinking back on when I began my

graduate career in 2012, there is absolutely no doubt in my mind that I would never have gained the knowledge necessary to be here today if it had not been for Dr. Nicolas DiLorenzo. He is one of my greatest friends and I hope that I can repay the enormous debt that I owe him someday.

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LIST OF ABBREVIATIONS

[H]	Metabolic hydrogen
0BSS	Basal substrate without bismuth subsalicylate in Chapter 2 – Experiment 3
5BSS	Basal substrate with 0.05% bismuth subsalicylate in Chapter 2 – Experiment 3
10BSS	Basal substrate with 0.10% bismuth subsalicylate in Chapter 2 – Experiment 3
33BSS	Basal substrate with 0.33% bismuth subsalicylate in Chapter 2 – Experiment 3
3-NOP	3-Nitrooxypropanol
A:P	Acetate to propionate ratio
APS	Adenosine phosphosulfate
BSS	Bismuth subsalicylate
BUN	Blood urea nitrogen
CAN	Calcium-ammonium nitrate
CAN1.2	Basal substrate with 0.38% urea and 1.2% CAN in Chapter 2 – Experiments 1 and 2
CAN2.4	Basal substrate with 2.4% CAN in Chapter 2 – Experiments 1 and 2
CO_2eq	Carbon dioxide equivalent
CTRL	Basal substrate with 0.75% urea and without calcium-ammonium nitrate in Chapter 2 – Experiment 1
eCAN	Encapsulated calcium-ammonium nitrate
EE	Ether extract
GHG	Greenhouse gas
GSH	Antioxidant glutathione
iNDF	Indigestible neutral detergent fiber

IVOMD	In vitro organic matter digestibility
MCR	methyl-coenzyme reductase
NCTRL	Basal substrate/diet without a non-protein nitrogen source
NIT	Basal diet with encapsulated calcium ammonium nitrate (350 mg/kg of body weight)
NITB	Basal diet with encapsulated calcium ammonium nitrate (350 mg/kg of body weight) and bismuth subsalicylate (58.4 mg/kg of body weight)
PEM	Polioencephalomalacia
SRB	Sulfate reducing bacteria
U	Basal diet with urea (182 mg/kg of body weight)
UB	Basal diet with urea (182 mg/kg of body weight) and bismuth subsalicylate (58.4 mg/kg of body weight)
UC	Ulcerative colitis

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON BEEF CATTLE PRODUCTION

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December 2017

Chair: Nicolas DiLorenzo
Major: Animal Sciences

To aid in the objective of becoming ever more efficient, scientists have developed strategies, such as providing nitrate as a NPN source, to produce high-quality beef and dairy products while mitigating the environmental impact of production. Another challenge in animal-protein production is elevated S concentrations in byproducts, forages, and water in certain areas of the U.S. Bismuth subsalicylate (**BSS**) has been observed to mitigate negative effects of S, such as H_2S, using ruminal in vitro systems. The overarching objective of this dissertation was to test the hypothesis that BSS and calcium-ammonium nitrate (**CAN**) would improve beef cattle performance and lessen the environmental impact. Four experiments were designed to evaluate BSS and CAN effects on in vitro and in vivo ruminal fermentation, enteric CH_4 production and nutrient digestibility, and on performance and liver mineral concentration. In vitro ruminal fermentation was altered by both BSS and CAN. Production of H_2S was reduced (P = 0.024) quadratically with the addition of up to 1.0% of the substrate DM. A linear reduction in total CH_4 ($P < 0.001$), CH_4 in mmol/g of substrate incubated ($P < 0.001$) and fermented ($P < 0.001$) was observed when CAN was provided up to 2.4% of the substrate DM. When provided to cattle in vivo, BSS had minimal effects on ruminal

fermentation; however, apparent S retention was reduced (P = 0.003) as was liver concentration of Cu (P = 0.002). In vivo, encapsulated CAN (**eCAN**) reduced ruminal total VFA concentration (P = 0.011), NH_3-N concentration (P = 0.001), apparent total tract DM and OM digestibility (P < 0.021), and enteric CH_4 production (g/kg of $BW^{0.75}$; P = 0.051) compared with urea. Performance of cattle was not affected (P > 0.05) by the addition of BSS or eCAN. As a potential NPN source, the data from this series of experiments indicate that eCAN may not promote the same ruminal fermentation and performance of cattle consuming urea. Bismuth subsalicylate appears to bind to S in the rumen; however, the negative effects on Cu concentration in the liver may impede upon further use of BSS in cattle fed low-quality forage-based, diets.

CHAPTER 1
REVIEW OF THE LITERATURE

Methane Production in Cattle

Methanogenesis

Methanogenesis in the rumen is a necessary process to prevent ruminal dysfunctions such as acidosis. As ruminal microorganisms degrade substrates, various redox reactions are occurring simultaneously, which alter and balance the concentration of ruminal metabolic hydrogen (**[H]**). These reactions involve many co-factors, which are continuously acting as a sink or donor of [H]. For example, when glucose is converted to pyruvate in glycolysis, glyceraldehyde-3-phosphate is oxidized to 1, 3-biphosphoglycerate, NAD^+ is reduced to NADH and H+ is released: G-3P + Pi + NAD^+ → 1, 3 BPG + NADH + H^+. There are a multitude of reactions such as this in the ruminal environment that transfer [H], which in turn prevents the pool size of H_2 from increasing to a point where acidosis becomes an issue (Janssen, 2010).

The ruminal pool of H_2 is warranting more attention (Janssen, 2010; Ungerfeld, 2015; Guyader et al., 2017) related to its effects on fermentation pathways in the rumen. The differences in ruminal fermentation between ruminants provided a high-grain vs high-forage diet are well recognized. For decades, it has been known that as readily digestible carbohydrates (i.e. starch) replace forages in ruminant diets, molar proportions of propionate increase while acetate molar proportions decrease (Balch and Rowland, 1957). In the past, it has been speculated that this shift in fermentation is simply due to shifts in populations of cellulolytic and amylolytic microorganisms. Recently, it has been postulated that this shift is more likely to be driven by ruminal concentration of H_2 (Janssen, 2010). A review by Janssen (2010), investigated the

notion that thermodynamics are controlling the use of these pathways. For example, when H_2 concentration is elevated in the rumen, non-H_2 producing pathways (0.66 acetate + 1.33 propionate) are more thermodynamically favorable. When ruminal H_2 concentration is lesser, the opposite is true; therefore, H_2 producing fermentation pathways (butyrate + 2 H_2 or 0.66 acetate + 0.66 butyrate + 2.66 H_2) are more thermodynamically favorable (Janssen, 2010).

The concept that shifts in fermentation are largely due to H_2 concentration is supported by diurnal shifts in molar proportions of VFA in the rumen. Concentration of H_2 has been shown to peak shortly after feeding for ruminants consuming forage- (Barry et al., 1977) and concentrate-based diets (Robinson et al., 1981). Furthermore, when Robinson et al. (1981) analyzed H_2 concentrations in the rumen of a cow consuming a concentrate-based diet, the authors observed that dissolved H_2 concentration was 15 μM at 1 h post feeding. The ruminal dissolved H_2 concentration reduced to a steady state 1 μM within 6 h post feeding (Robinson et al., 1981). With this shift in dissolved H_2 concentration, thermodynamics of fermentation are also going to shift, i.e. shift from H_2 producing pathways to non-H_2 producing pathways (Janssen, 2010). Ciriaco et al. (2016) provided ruminally cannulated steers with bermudagrass hay and supplemental 50:50 molasses:glycerol mixture. When evaluating the VFA profile, a reduction in acetate molar proportion after feeding the liquid feed (9% reduction 3 h post feeding) was observed, returning to pre-feeding proportions 9 h post feeding (Ciriaco et al., 2016). It is unlikely that shifts in microbial populations occur this rapidly (within hours) to alter fermentation pathways. What is more probable is that when the supplement was

provided, H_2 concentration in the rumen may have peaked, causing acetate forming pathways to become thermodynamically unfavorable (Janssen, 2010).

When investigating the impact of H_2 concentration on methanogenesis, it is pertinent to consider not only the pool of H_2, but also the flux within the pool (Janssen, 2010). The rate at which CH_4 is produced is directly related to the rate at which H_2 passes through the pool of dissolved H_2 and the amount of CH_4 produced is determined by the amount of H_2 that passes through the pool (Janssen, 2010). Again, when H_2 concentration is elevated (as with concentrate diets), production of H_2 (i.e. the flux of H_2 through the pool of dissolved H_2) is reduced, which in turn reduces the rate and amount of CH_4 produced per unit of substrate degraded by ruminal microorganisms (Janssen, 2010).

Issues Related to Enteric Methane Production

The ruminant is a very effective instrument for producing the world's protein supply through meat and milk. It has been reported that of the total solar energy captured by biomass on earth, only 5% is potentially available for human consumption (Russell and Gahr, 2000). The majority of the remaining 95% can be consumed by ruminants, then fermented and enzymatically broken down by ruminal microorganisms into products such as VFA, which are then used as energy to produce meat and milk (Russell and Gahr, 2000). One disadvantage of the fermentation of substrates to VFA is the production of reducing equivalents, which, in turn, need to be disposed of. Disposal of reducing equivalents is performed by transferring electrons and H_2 to various acceptors in the rumen. Methane, which is formed by the reduction of CO_2 or formate, is one of the most common and important hydrogen sinks in the rumen (Russell and Gahr,

2000). Methanogens, a subgroup of *Archaea*, use hydrogen as an energy source, and primarily, CO_2 as a carbon source to form CH_4. The hydrogen-consuming process of methanogens reduces the concentration of hydrogen in the rumen to allow for rapid fermentation of substrates (Buddle et al., 2011).

Regardless of the importance of CH_4 as a hydrogen sink in the rumen, the production of CH_4 can be detrimental to the environment by adding to the already elevated concentration of greenhouse gases (**GHG**) in the atmosphere. In general, GHG are measured in equivalents of CO_2 (**CO_2eq**) and CH_4 is considered to be 20-25 times more potent as a GHG than CO_2 (FAO, 2013). Enteric CH_4 production in ruminants varies among diets of differing levels of concentrate, digestibility, and other aspects. In general, high-forage diets produce 3 to 4-fold more CH_4 than high-concentrate diets (Johnson and Johnson, 1995). Nearly 70% of the energy requirements for cattle are used prior to weaning, when the primary dietary component is forage (Shike, 2013); therefore, reducing enteric CH_4 production is a vital step in providing enough animal protein for the growing population while preserving our environment. Life cycle assessments investigating various cattle production systems have reported that the largest influence on the carbon footprint of beef and dairy production is represented by on-farm emissions, when cattle are generally grazing (Beauchemin et al., 2010; Kristensen et al., 2011). When evaluating beef production specifically, approximately 80% of GHG emissions are released from the cow-calf phase of production (Beauchemin et al., 2011). It has been estimated that an increase, greater than 6%, in global enteric CH_4 production will occur between the year 2015 to

2020 (2204 to 2344 $MtCO_2eq$, respectively; EPA (2006). This is a 32% increase from the year 1990 (EPA, 2006).

Methane not only has an effect on the environment, but also on energy losses in cattle. It has been reported that CH_4 accounts for up to 12% of GE losses in cattle (Johnson and Johnson, 1995). The percentage of energy lost is highly related to the type of diet provided. Losses from forage-based diets typically are 2-fold greater than energy losses to CH_4 from cattle consuming high-concentrate diets (Harper et al., 1999; Doreau et al., 2011).

Mitigation of Enteric Methane Production

Many researchers have investigated the mitigation of CH_4, and potential strategies are as follows: 1) increase feed utilization efficiency by improving the digestibility of forages and feedstuffs; 2) develop feed additives to act as H_2 sinks in the rumen; 3) reduce or inhibit methanogen populations in the rumen; 4) provide substrate to microorganisms other than methanogens to aid in the formation of more efficient products, such as propionate; and 5) improve the efficiency of production (meat, milk, etc.) to reduce the number of livestock required (USDA, 2004).

Tannins

Tannins are a group of diverse polymeric flavonoids with either C-C or C-O-C bonds and are principally found in the cell walls or vacuoles of dicotyledonous plants (McMahon et al., 2000). Tannins can improve performance of ruminants consuming diets with CP concentrations above nutritional requirements by binding to excess protein allowing for post-ruminal digestion. In contrary, if CP is limiting, detrimental effects to the animal, related to reduced amino acid absorption, may be observed (Waghorn, 2008).

Throughout the literature, there are several different sources of tannins used to alter fermentation of ruminants. These tannins vary widely in their effectiveness of CH_4 mitigation and impact on animal performance. Within tannin groups, there is also large variability. For example, quebracho tannin has been fed to beef, dairy, and boer goats, as well as used in in vitro experiments with differing results (Pellikaan et al., 2011; Dickhoefer et al., 2016; Ebert et al., 2017). Pellikaan et al. (2011) compared several different tannins on in vitro fermentation of lucerne hay (*Medicago sativa*). The authors observed that CH_4 as a percent of total gas production and rate of CH_4 production was reduced with the addition of quebracho tannin (Pellikaan et al., 2011). Conversely, Ebert et al. (2017) provided quebracho tannin to growing beef steers (350 ± 32 kg BW) consuming a 92% concentrate finishing diet and observed no change in enteric CH_4 production. The differences observed between the results of Ebert et al. (2017) and Pellikaan et al. (2011) could be related to differences in in vitro and in vivo experiments or the diet provided. However, when quebracho tannin was provided to growing beef heifers (238 ± 13 kg BW) consuming a 70% barley silage diet, no change in enteric CH_4 emissions compared with control heifers was observed (Beauchemin et al., 2007). Furthermore, Beauchemin et al. (2007) reported a 15% reduction in apparent total tract digestibility of CP when quebracho tannin was provided at 2% diet DM.

Other sources of tannins have presented different results from quebracho. Puchala et al. (2005) provided Angora does with either a crabgrass (*Digitaria ischaemum*)/tall fescue (*Festuca arundinacea*) mixture or sericea lespedeza (*Lespedeza cuneata*). The sericea lespedeza contained 18% condensed tannins whereas the crabgrass/tall fescue mixture contained 0.5%. Differences between the

forages, with in vitro DM digestibility being 64.5 and 75.3% for sericea lespedeza and crabgrass/tall fescue, respectively, were observed. However, the researchers reported a 50% reduction in CH_4 as grams per kilogram of digestible DMI along with a 40% increase in DMI for the does consuming sericea lespedeza, when compared with the crabgrass/tall fescue fed does (Puchala et al., 2005).

Using 60 Holstein-Friesian cows (40 cows for CH_4 measurements), Grainger et al. (2009) evaluated the effects of *Acacia mearnsii* tannin on enteric production of CH_4 and nitrogen excretion. Cows were grazing a mixture of diploid and tetraploid ryegrass hybrids (*Lolium* × *boucheanum* syn. *L. hybridum*) and were supplemented with 4.5 kg/d of cracked triticale (*Triticosecale*) grain. Cows were dosed with either 163 or 326 g/d of tannins. Grams of CH_4 per d were significantly reduced when cows received tannins extracted from the bark of *A. mearnsii*; however, when emission intensity was considered using grams of CH_4 per kg of fat + protein yield in milk, the researchers observed no differences between treatments. This was due to the 12% reduction in milk fat + protein yield when tannin was dosed up to 326 g/d. Until researchers can find suitable tannins to reduce CH_4 production, without negatively affecting production of the animal, other routes of mitigation should be sought after.

Lipids

Lipid supplementation has negative impacts on the activity of methanogens and ruminal protozoa (Johnson and Johnson, 1995). Saturated fatty acids, from sources such as coconut oil (lauric acid; C12:0), have toxic effects on ruminal protozoa and methanogens (Zhou et al., 2013); however, it appears that unsaturated fatty acids can be detrimental to methanogenic archaea as well (Prins et al., 1972). Researchers have also described a process in which electrons are accepted during biohydrogenation

within the rumen, lessening the availability of H_2 for methanogens to use as an energy source (Boadi et al., 2004). Moreover, lipid supplementation can decrease CH_4 production by limiting fermentation of OM due to a physical coating of the feed particles, inhibiting bacteria to attach and degrade the material (Johnson and Johnson, 1995). Nevertheless, an issue associated with lipid supplementation is that fats should not be provided at concentrations greater than 5 to 6% of the diet DM due to negative effects on digestibility of fiber (Boadi et al., 2004) and milk fat production (Jenkins et al., 1996; Ashes et al., 1997).

To evaluate differing lipid sources, a research group designed an experiment using 45 Nellore steers (419 ± 11 kg BW; 20 steers for CH_4 emissions) consuming a 60:40 forage:concentrate ration for 118 d (Fiorentini et al., 2014). The steers were randomly assigned to 1 of 5 treatments: control (2.8% ether extract; **EE**), control + palm oil (7.1% EE), control + linseed oil (7.0% EE), control + protected fat (7.0% EE), and control + whole soybean (7.0% EE). Lipid supplementation impacted DMI of the Nellore steers, with control having greater DMI compared with palm oil (46% reduction), linseed oil (20% reduction), and whole soybean (27% reduction) addition (Fiorentini et al., 2014). Control and protected fat fed steers had similar DMI. Protected fat did not have any effect on enteric CH_4 production as would be expected, since the purpose of protecting fats is to allow passage out of the rumen without being affected by the ruminal microorganisms. All other sources of lipid provided resulted in reductions in total CH_4 production (grams per d; 56% reduction) and CH_4 produced as a percentage of GE intake (33% reduction). Supplementation of lipids (not including protected fat) did

reduce performance of the steers with ADG being reduced by 41% (Fiorentini et al., 2014).

A meta-analysis was conducted to evaluate CH_4 mitigation by lipid supplementation of lactating dairy cows using data from 7 publications, resulting in 25 diets (Eugène et al., 2008). The authors reported a 9% overall reduction in enteric CH_4 production (MJ/d) when the diets contained an average of 6.4% ether extract. This reduction in CH_4 production was attributed to a reduction (6.5%) in DMI of the cows across the 25 diets. When performance was evaluated, the authors observed that 4% fat corrected milk was unchanged by the addition of lipids to the diets; therefore, enteric CH_4 emission intensity, in terms of MJ per kilogram of 4% fat corrected milk, was reduced by 9% (Eugène et al., 2008).

Zhou et al. (2013) used pure cultures of *Methanobrevibacter ruminantium* to elucidate the mode of action of saturated fatty acids on inhibition of methanogenesis. *Methanobrevibacter ruminantium* was incubated with 10 µg/mL of lauric (C14:0), myristic (C14:0), palmitic (C16:0), or stearic (C18:0) acid. The authors reported greater impacts on CH_4 production with the shorter chain fatty acids. Furthermore, it was observed that cell viability (ratio of live vs. dead cells) was reduced and extracellular K^+ concentration increased with lauric and myristic acid. The resulting increase in extracellular K^+ concentration indicates cell membrane permeation which can lead to cell death, thus reducing methanogenic capability of *M. ruminantium* (Zhou et al., 2013).

Ionophores

Ionophores are lipophilic substances that alter the flow of ions across the membranes of certain bacteria eventually de-energizing the cell, causing necrosis (DiLorenzo and Galyean, 2010). Although several ionophores, such as lasalocid and

laidlomycin (Bartley et al., 1979; Domescik and Martin, 1999; Appuhamy et al., 2013), have been investigated in ruminants with reductions in enteric CH_4 production, monensin has been investigated more thoroughly than other ionophores and is used on a daily basis in beef and dairy production in the U.S. (FAO, 2013); therefore, the majority of data discussed in this sub-section will focus on monensin.

Monensin has been used for decades in the beef and dairy industry (Goodrich et al., 1984; Duffield et al., 2008). It has been reported that monensin can reduce the acetate to propionate ratio (**A:P**; Bogaert et al., 1990; Guan et al., 2006; Ellis et al., 2012) and reduce protozoa populations in the rumen (Hino and Russell, 1987; Guan et al., 2006). With these changes in ruminal fermentation, the potential for mitigating methanogenesis has been investigated.

A group of researchers performed a meta-analysis evaluating the anti-methanogenic effects of monensin on beef and dairy production systems (Appuhamy et al., 2013). This meta-analysis encompassed 13 manuscripts (6 dairy and 7 beef related) with 22 experiments (11 dairy and 11 beef related). The average monensin dose was 21 and 32 mg/kg of DMI, and was provided for an average of 72 and 38 d for dairy cows and beef steers, respectively. Total CH_4 production (grams per d) was reduced 15% and CH_4, as a percentage of GE lost, was reduced by 9% for beef steers provided monensin (Appuhamy et al., 2013). The reduction in total CH_4 produced could be partly attributed to a reduction in DMI (-0.41 kg reduction) of the steers; however, when evaluated as a percentage of GE lost, DMI did not account for any reduction in CH_4 production. Enteric CH_4 production was not affected by monensin when provided to dairy cows at an average rate of 21 mg/kg of DMI; however, DMI was reduced without a

negative effect on milk yield leading to the probability that emission intensity was reduced by the addition of monensin (Appuhamy et al., 2013).

It has been proposed that although intake, shifts in VFA profile, and feed efficiency of beef cattle consuming monensin are persistent over time, enteric CH_4 mitigation may not persist for the entire time that monensin is provided (Guan et al., 2006). To investigate this, researchers provided 36 Angus steers (328 ± 25 kg BW) either a 75:25 or 30:70 forage:concentrate ration for 16 wk. Within the 2 groups of steers, 3 treatments were randomly assigned: control (no ionophore), control + monensin (33 mg/kg diet DM), and control + monensin/lasalocid (lasalocid was provided at 36 mg/kg diet DM; steers were alternated every 2 weeks from monensin to lasalocid). The authors reported no effects of treatment on DMI for the 75:25 ration; however, DMI was reduced for steers consuming ionophores compared with steers in the control group consuming the 30:70 ration. This reduction in DMI was accompanied by no change in performance, which led to an increase in feed efficiency. Using the SF_6 tracer technique, the researchers assessed enteric CH_4 production at wk 1, 3, 4, 5, 6, 8, 10, 12, and 16. When evaluating CH_4 as a percentage of GE intake, the authors observed marked reductions for ionophore treated cattle at wk 3 and 4 for the high-concentrate diet; however, the high-forage diet maintained the reduction in CH_4 production from wk 3 to 6. By wk 8, all treatments returned to baseline values. To further investigate this short-term effect of ionophores on enteric CH_4 production, the authors measured protozoa counts and observed that protozoa in the rumen were reduced during the same wk that CH_4 was reduced. These data imply that the reduction in CH_4, not accounted for by DMI, is likely due to inhibition of protozoa growth in the rumen (Guan

et al., 2006). Furthermore, these data indicate that persistency of CH_4 mitigation may not last longer than 6 wk, which could explain variability in CH_4 emissions found in the literature.

3-Nitrooxypropanol

A promising molecule that has gained the regard of ruminant nutritionists is known as 3-nitrooxypropanol ($C_3H_7NO_4$; **3-NOP**). This small molecule impedes methanogen growth in the rumen by inhibiting the reduction of CO_2 to CH_4 (Duin et al., 2016). In the process of reducing CO_2 to CH_4, methyl-coenzyme reductase (**MCR**) is a necessary catalyst. A tetrapyrrole derivative named cofactor F_{430} is a portion of the nickel enzyme MCR and it is where nickel is bound. For F_{430} to be active, the nickel has to be in the Ni(I) form. Acting as an oxidant, 3-NOP, which has a similar shape as MCR, binds and inactivates MCR, halting the formation of CH_4 (Duin et al., 2016). Several research groups have reported significant reductions in enteric CH_4 production of large and small ruminants treated with 3-NOP (Haisan et al., 2014; Martínez-Fernández et al., 2014; Reynolds et al., 2014).

Vyas et al. (2016) used 84 crossbred steers (319 ± 20 kg BW) in a 238-d feeding trial. For the first 105 d (backgrounding phase), steers were provided a barley silage-based backgrounding diet followed by 28 d of adaptation to a dry-rolled barley grain-based diet, which was provided for 105 d (finishing phase). Steers were randomly assigned to 1 of 3 treatments: control (no addition of 3-NOP); low 3-NOP (100 mg/kg of diet DM); or, high 3-NOP (200 mg/kg of diet DM). To determine enteric CH_4 emissions, 15 steers (5 per treatment) were evaluated using calorimetry chambers. During the backgrounding phase, there was a reduction in DMI for steers consuming high 3-NOP when compared with control and low 3-NOP steers; however, grams of CH_4 per

kilogram of DMI was also reduced for high 3-NOP (Vyas et al., 2016). The authors attributed the hypophagic effects of 3-NOP to a possible increase in ruminal propionate molar proportions. Researchers have observed significant alterations in the ruminal VFA profile of ruminants treated with 3-NOP (Haisan et al., 2014; Martínez-Fernández et al., 2014; Romero-Perez et al., 2014). It is evident that 3-NOP generally increases propionate molar proportions leading to a reduction of A:P. The shift in molar proportions of VFA could be due to an increase in ruminal H_2 concentration, which would transfer [H] from H_2 forming (2 acetate + 4 H_2) to non-H_2 forming pathways (0.66 acetate + 1.33 propionate; Janssen, 2010). Throughout the finishing phase, DMI was reported to be reduced for steers consuming high 3-NOP, which lead to a tendency for a reduction of ADG for high 3-NOP as well (Vyas et al., 2016). Methane emission intensity was reduced by 81% for high3-NOP compared with control steers (Vyas et al., 2016).

Other researchers focusing on 3-NOP have also reported reduction of enteric CH_4 production in ruminants with improvements in performance indices. Haisan et al. (2014) observed a 60% reduction in grams of CH_4 per kilogram of DMI when 2,500 mg/d (128 mg/kg diet DM assuming 19.5 kg DMI) of 3-NOP was provided to Holstein cows (580 ± 76 KG BW) consuming barley silage- and ground corn-based diet. This reduction in CH_4 was accompanied by an increase in ADG of the cows without altering DMI or milk production (Haisan et al., 2014). Similarly, researchers using 48 Holstein cows (653 ± 12 kg BW) consuming a 50:50 forage:concentrate diet observed a 29% decrease in CH_4 (g/d) with a 36% increase in ADG for cows consuming 80 mg/kg diet DM compared with control cows.

Diet

Another effective, but not always viable, strategy for mitigating CH_4 is by altering the composition of the diet. The composition of the diet, specifically the types of carbohydrates used, has a great impact on CH_4 production due to its effects on ruminal pH, concentration of H_2, and subsequent consequences on the ruminal milieu (Johnson and Johnson, 1995). Cellulose and hemicellulose digestibility have a greater correlation with CH_4 production when compared to soluble carbohydrates (Hook et al., 2010). Furthermore, by increasing starch concentration, decreasing cell wall components, and processing the ingredients in the diet, ruminants will encounter increased passage rate, which is associated with an increase in H_2 concentration, reduction of A:P, and possible reduction of the ruminal pH (Owens et al., 1998; Janssen, 2010). With these alterations in ruminal fermentation, CH_4 production is likely to decrease (Janssen, 2010); however, the risk of sub-acute ruminal acidosis increases (Owens et al., 1998). Shifting fermentation pathways to propionate production likely is caused by greater concentrations of H_2, which in turn makes H_2 producing pathways thermodynamically less favorable. A decrease in CH_4 production will occur since the flux of H_2 through the pool will be reduced, whereas the opposite is true for substrates (high-cellulose containing diets) that produce less H_2 during degradation and, therefore, shift to more thermodynamically favorable pathways, acetate and butyrate (Van Nevel and Demeyer, 1996; Janssen, 2010).

Using 24 growing *Bos taurus* crossbred heifers (318 ± 35 kg BW) in two periods (n = 48), an experiment was designed to investigate the effects of chitosan (Henry et al., 2015), a natural bio-polymer with anti-microbial activity (Chung et al., 2004; Qi et al.,

2004; Daetz et al., 2016). The heifers were randomly assigned to 1 of 6 treatments arranged as a 2 × 3 factorial with diet (64:36 and 15:85 roughage:concentrate) and chitosan inclusion rate (0.0, 0.5, and 1.0% diet DM) being the factors. Chitosan had no impact on enteric CH_4 production; however, when the main effect of diet was considered, heifers consuming the high-roughage diet produced nearly 5-fold more CH_4, as grams per kilogram of DM digested, when compared with heifers consuming the low-roughage diet.

As a result of the dependence of grazing forages with little supplementation, it is difficult to alter ruminal fermentation by modifying the diet of cattle in the cow-calf sector of the U.S.; hence, there is a need for a mitigation strategy that can be applied to the 30.3 million head of beef cows grazing forages in the U.S.

Potential for Nitrates in the Cattle Industry

For several years, nitrate has been considered as a tool to inhibit enteric CH_4 production of ruminants (Sar et al., 2004; Leng, 2008); however, there has been political and social concerns related to toxicity associated with nitrite accumulation in the rumen. Nitrite can be absorbed through the rumen epithelium into the blood stream where it converts hemoglobin to methemoglobin. When methemoglobin concentration is greater than 30% of the total hemoglobin, issues with transporting O_2 to tissues can be observed (Benu et al., 2016).

Researchers have determined that microbial populations that reduce nitrate to nitrite have a rapid growth rate, whereas nitrite reducers have a slow growth rate leading to an accumulation of nitrite in the rumen if the microbiome is not properly adapted to nitrate inclusion (Leng, 2008). To hinder this concern, adaptation to nitrate can be achieved by providing increasing amounts over an adaptation period (Newbold

et al., 2014). Through adaptation, researchers have reported supplementing nitrate to cattle at up to 3.0% diet DM (Newbold et al., 2014).

Mitigation of enteric methane production with nitrate

Nitrates have been provided to sheep and cattle, mitigating enteric CH_4 production between 15 and 30% (van Zijderveld et al., 2010; van Zijderveld et al., 2011; Hulshof et al., 2012). Most of the research regarding nitrate as an inhibitor of CH_4 has utilized high-concentrate diets (feedlot animals) or diets with at least 50% concentrate (backgrounding and dairy animals). Very little research has focused on the effects of nitrate on enteric CH_4 production and performance of cattle consuming forage-based diets (Hulshof et al., 2012; Benu et al., 2016).

Methane production decrease has been proposed to be due to the reduction of nitrates to nitrites, and finally, to NH_3 (FAO, 2013), which is thermodynamically more favorable than the reduction of CO_2 to CH_4 (Ungerfeld and Kohn, 2006). Certain bacteria within the rumen are very efficient at reducing nitrates. *Denitribacterium detoxificans* and *Wolinella succinogenes* utilize nitrates within the rumen, and *Wolinella succinogenes* has been shown to rapidly reduce nitrate to NH_3 with little accumulation of nitrite (Morgavi et al., 2010). It has been reported that increasing the number of nitrate reducing bacteria in the rumen decreases CH_4 production when nitrate is available for reduction (Morgavi et al., 2010). Nitrate can be reduced by 3 pathways: denitrification, which leads to N_2O, assimilatory, and dissimilatory nitrate reduction to NH_3. Although N_2O has been detected with the addition of nitrates (Latham et al., 2016), researchers have observed that only 0.02% of nitrate is converted to N_2O in vitro (Guyader et al., 2017). Furthermore, assimilatory reduction of nitrate to NH_3 is an energy consuming pathway that is suppressed by NH_3, whereas the dissimilatory

pathway produces energy; therefore, it is more favorable for ruminal microorganisms to reduce nitrate using the dissimilatory pathway (Leng, 2008; Latham et al., 2016).

Nitrate may act as a H_2 sink in the rumen, but other modes of action for its anti-methanogenic activity have been investigated. The addition of nitrate has been reported to reduce populations of methanogens by up to 97% in vitro (Zhou et al., 2012). This reduction in CH_4 producing archaea has been shown to be due to the effects of nitrite on methanogens. Recently, while elucidating the mode of action of 3-NOP, Duin et al. (2016) reported that nitrite completely inhibits MCR. It would be assumed that nitrate, acting as a [H] sink, would reduce the concentration of H_2 in the rumen; however, there is contradicting research that reports H_2 to increase with the addition of nitrate (Guyader et al., 2015). To speculate, this may be related to the inhibitory action of nitrite on the reduction of CO_2 to CH_4 by ruminal methanogens, which perhaps, could increase ruminal H_2 concentration to a great enough extent that nitrate could not capture all of the [H].

Nitrate as a non-protein nitrogen source

For nitrate to not only have beneficial effects towards the carbon footprint of the cattle industry, it must also have characteristics that will provide cattle producers with incentive to provide nitrate to their cattle. One possibility is to utilize nitrate as a NPN source to cattle consuming low-nitrogen content forages or diets.

To evaluate the effect of nitrate on 300 growing Nellore bulls (392 ± 28kg BW), Newbold et al. (2014) included incremental amounts of nitrate (0.0 to 2.4% diet DM) into a 16:84 forage:concentrate diet. The bulls were randomly assigned to 1 of 6 treatments (0.0, 0.48, 0.96, 1.44, 1.92, or 2.40% nitrate in the diet DM) made isonitrogenous with urea, and fed for 111 d. There was no effect of nitrate on final BW, ADG, or HCW;

however, DMI was linearly reduced as the inclusion of nitrate increased to 2.4% diet DM. The researchers also observed a 9% increase in G:F when comparing the 0.0% with 2.4% nitrate diets.

An experiment was designed with the objective of determining the effects of nitrate, in place of urea, provided to 432 (435 kg BW) composite-breed steers fed a 12:88 forage:concentrate ration for 108 d (Hegarty et al., 2016). Steers were assigned to 1 of 4 treatments that were arranged in a 2 × 2 factorial with NPN source (urea or nitrate) and inclusion rate of NPN (0.25 or 0.45%) being the factors. Steers that were provided nitrate, regardless of inclusion rate, had lesser ADG compared to cattle supplemented urea as a NPN source. This reduction in ADG led to a reduction in HCW for nitrate fed steers compared with urea. Furthermore, the ADG was likely affected by the 600 g reduction in DMI of steers provided nitrate compared with urea. The authors reported no effects of treatment on carcass traits or nitro-compounds in the meat (Hegarty et al., 2016).

It is evident that further research is needed to determine the efficacy of supplementing nitrate as a NPN in place of urea. Additionally, very little research on the use of nitrates in a high-forage or grazing system has been performed. Many areas in the U.S., such as the southeast, have forages that lack sufficient nitrogen content for not only the animal, but also production of ruminal microorganisms. It would be valuable to U.S. producers to be able to provide a NPN to cattle grazing low-protein forages while reducing the impact of their operations on the environment.

Sulfur in Beef Cattle Production

Issues Related to High-Sulfur Diets

In the rumen, H_2S is predominantly formed by dissimilatory sulfate reducing bacteria (**SRB**) (Drewnoski et al., 2014). Briefly, sulfate is transported into the cell via sulfate permease, where ATP sulfurylase activates the sulfate. Activated sulfate can then re-activate adenosine phosphosulfate (**APS**). Reduction of APS to sulfite occurs through catalyzation via APS reductase (Bradley et al., 2011). For the reduction from sulfite to H_2S, there are multiple proposed models (Kobayashi et al., 1969; Rees, 1973). The Rees (1973) model proposes that sulfite is directly reduced to H_2S via dissimilatory sulfite reductase, whereas Kobayashi et al. (1969) offered a model where intermediary products, such as trithionate, were produced and further reduced to H_2S.

It is likely that H_2S toxicity occurs due to the inhalation of eructated ruminal H_2S (Gould, 1998). Therefore, mitigation of ruminal production of H_2S is key to reducing toxicity. Concentration of H_2S in the gas cap of the rumen is predominantly determined by sulfide production in the ruminal fluid (pKa of H_2S = 7.04), pH of the rumen, frequency of eructation, and absorption of sulfide through the ruminal mucosa (Gould, 1998).

It has been agreed upon that H_2S is the culprit in the neurological disorder polioencephalomalacia (**PEM**; Gould, 1998; Cammack et al., 2010; Drewnoski et al., 2014). In ruminants, PEM is a neurological disease with symptoms similar to “blind staggers” where lesions of the grey matter can be found along with gross and microscopic features not recognized for a particular etiology (Gould, 1998). Incidence of PEM when high-S diets are consumed is around 1% (Nichols et al., 2012). Concentrations of H_2S greater than 2000 mg/L have been implicated in PEM; however,

studies have reported concentrations 2 to 3-fold greater with no symptoms of PEM (Drewnoski et al., 2012; Drewnoski et al., 2014).

Cytotoxicity due to elevated concentrations of H_2S may be explained in various ways (Drewnoski et al., 2014). Hydrogen sulfide can easily and hastily cross lipid bilayers, where it can inhibit cytochrome c oxidase, the last enzyme in the electron transport chain (Truong et al., 2006). It has been reported that H_2S also depletes the antioxidant glutathione (**GSH**). Using hepatocytes, researchers reported that a source of H_2S reduced the amount of GSH in the cell, eventually leading to a rapid necrosis, likely related to the involvement of reactive S and O species (Truong et al., 2006). Intracellular Fe stores are released from ferritin proteins due to increased H_2S concentrations, respiration is inhibited, super oxide ions, and subsequently H_2O_2, are accumulated, ATP is depleted, anaerobic metabolism increases and cellular pH decreases, eventually causing cell death (Truong et al., 2006).

Adequate mineral nutrition is a component of successful cattle performance, not only due to production indices, but also the biological roles in which minerals have a significant responsibility (NASEM, 2016). Elevated concentrations of S can interfere with trace mineral absorption. The NASEM (2016) recommends concentrations of S in the diet to be at least 0.15 and no more that 0.4% of the diet DM. It is likely that the concentration of S that interrupts trace mineral absorption is lower than the concentration in which issues with cattle performance become evident (Drewnoski et al., 2014). When sulfur binds to molybdate in the rumen, it can form complexes called thiomolybdates, which along with sulfide, binds Cu inhibiting its absorption and subsequent use by the animal (Suttle, 1991). In the U.S., many beef cow herds are

provided with some type of forage during the winter months, such as hay or planted winter forage, and very often, a high-S supplement, such as corn gluten feed. It is less likely that these females will develop PEM on a high forage diet; however, inhibition of trace mineral absorption should be considered to ensure proper growth and health (Drewnoski et al., 2014).

The effect of S concentration in the diet on DMI of growing cattle is more likely to be affected by S concentrations in a high-concentrate rather than a high-forage diet (Spears et al., 2011; Drewnoski et al., 2012). This tolerance to elevated S in forage-based diets may be related to increased pH of the rumen and/or the ruminal microorganisms present in a forage fed animal (Drewnoski et al., 2014). Another consideration is the type of S provided to the animal. It has been reported that cattle consuming rations with more than 0.2% inorganic S have decreased DMI, ADG, and HCW (Drewnoski et al., 2014).

A major provider of S to feedlot diets is distiller's grains. When distiller's grains were included in feedlot rations, up to 30% of the diet DM, there was an increase in ADG; however, when surpassing 30% ADG began to decline (Klopfenstein et al., 2008). It has been suggested that due to possible negative effects on DMI and ADG, to achieve a desired HCW when providing a high-S diet, cattle may need to stay on feed for longer than anticipated. Additionally, when S content was 0.46% of the diet DM, LM area was decreased compared to cattle consuming diets with less than 0.2% S. Few investigators have considered the effects of H_2S on performance indices. A negative correlation was reported for H_2S concentrations and ADG and DMI (Uwituze et al., 2011).

Mitigation of Sulfur Toxicity

The availability of S in the rumen may have a significant impact on S toxicity. A concept known as adjusted ruminal protein S proposes that S from differing sources differs in their availability within the rumen (Sarturi et al., 2013). This concept comes from the idea that S from RUP is unavailable to SRB of the rumen. For example, of the S in wet distiller's grains, only 71% is expected to be ruminally available, whereas 100% of the S from inorganic sources, such as ammonium sulfate, are available to SRB (Sarturi et al., 2013).

Concentration of fiber in the diet can have positive effects on the risk of S toxicity. A meta-analysis of feedlot data described a 19% decrease in the risk of PEM for every 1% increase of NDF (Nichols et al., 2012). In accordance, investigators have observed that increasing NDF significantly decreases H_2S production (Morine et al., 2014a) without having effects on the performance of growing cattle (Morine et al., 2014b). Ruminal pH is generally elevated in cattle consuming high-forage diets compared to those consuming high-concentrate diets (Counotte et al., 1979). The increase in pH may inhibit H_2S production to some degree due to the pKa (7.04) of H_2S (Gould, 1998; Drewnoski et al., 2014). Some research has indicated a negative correlation between ruminal pH and H_2S when pH of the rumen is less than 5.8; however, at a ruminal pH greater than 5.8, ruminal pH is a poor predictor of H_2S production (Morine et al., 2014b).

Mitigation of Hydrogen Sulfide Production with Bismuth Subsalicylate

In humans, H_2S has been implicated as a bacterial product that could damage the gut mucosa (Mitsui et al., 2003). Production of excess H_2S in the large intestine may be pathogenic causing ulcerative colitis (**UC**), a form of inflammatory bowel disease that is known for ulcers and open sores in the colon (Levine et al., 1998). In the past two

decades, researchers have investigated the use of bismuth to aid UC. Although the mechanisms of bioactivity have not been fully elucidated for bismuth, it has been suggested, due to its thiophilic behavior, that biological molecules containing S (i.e. thiol- or thiolate-) are likely candidates for bismuth binding (Phillips et al., 2007). When bismuth interacts with sulfide, it reacts to form an insoluble black salt called bismuth sulfide. This is believed to be the cause of black feces observed in patients treated with bismuth subsalicylate (**BSS**; Suarez et al., 1998). Additionally, bismuth, in the form of BSS, has been shown to have antimicrobial effects against *Helicobacter pylori*, a bacterium known for causing ulcers in the gastro-intestinal tract of humans, and *E. coli* (Manhart, 1990; Suarez et al., 1998).

Greater numbers of SRB can be found along with increased H_2S production in fecal samples of humans with UC as compared to healthy patients (Levine et al., 1998; Pitcher et al., 2000). Through the use of popular over-the-counter medicines, such as Pepto-Bismol (262 mg of BSS per caplet), to aid in the gastrointestinal issues, the importance of bismuth in medicine is underscored (Phillips et al., 2007). In the laboratory, when BSS was fixed in a gas space with H_2S, BSS quickly removed H_2S by binding to the S (Suarez et al., 1998). To determine whether subsalicylate had any effect on H_2S, researchers designed an experiment using bismuth acetate and sodium salicylate. It was reported that bismuth acetate rapidly bound to H_2S, whereas sodium salicylate had no effect (Suarez et al., 1998). In fecal slurries, H_2S was reduced up to 90% when bismuth was included (Mitsui et al., 2003). Moreover, results from an in vivo study indicated that humans treated with BSS had virtually no H_2S production in feces

for up to seven days of treatment. This reduction in H_2S no longer existed after 5 days without BSS (Suarez et al., 1998).

Similar to human trials, H_2S production from feces was significantly reduced when rodents were treated with BSS (Suarez et al., 1998; Levitt et al., 2002). In addition, when cecum concentrations of H_2S were considered, rats consuming rat chow with BSS had reduced production compared to non-treated rats (Suarez et al., 1998). Researchers have recognized BSS as a tool to quantify H_2S production, through the quantification of bismuth sulfide, due to the innate ability of BSS to bind to sulfur in H_2S (Levitt et al., 2002).

Few investigations of BSS on ruminal fermentation have been made through in vitro fermentations using ruminal fluid as an inoculum (Ruiz-Moreno et al., 2015). When BSS was added to a high-S substrate (0.44% S, DM basis) at 1.0% of the diet DM, H_2S concentration was decreased, as was A:P, without affecting total VFA production (Ruiz-Moreno et al., 2015). No changes occurred for NH_3-N. When BSS was added to a comparable substrate at 1.0% diet DM in continuous culture system, H_2S was reduced by 98% as compared to control substrates (Ruiz-Moreno et al., 2015). In the continuous culture system, BSS significantly increased degradability of NDF and ADF (Ruiz-Moreno et al., 2015). In contradiction, VFA production was reduced and A:P was increased. Furthermore, NH_3-N was increased when BSS was included (Ruiz-Moreno et al., 2015). The opposing results from the two in vitro studies for VFA and NH_3-N production along with coinciding H_2S results illustrates a need for further research into this potentially industry changing bismuth compound.

CHAPTER 2
EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON IN VITRO FERMENTATION OF BAHIAGRASS HAY WITH SUPPLEMENTAL MOLASSES

Introduction

Many researchers have focused on reducing enteric CH_4 production through dietary modification (Shibata et al., 1992; Mc Geough et al., 2010), inhibition of methanogens (Prins et al., 1972; Zeitz et al., 2013), and providing H_2 sinks within the rumen (van Zijderveld et al., 2010; Newbold et al., 2014; Lee et al., 2015a). Much of the research has been focused on enteric CH_4 production of ruminants consuming diets which contain at least 50% concentrate. It has been determined that 80% of the greenhouse gas (**GHG**) emissions of beef production, in kg of CO_2 equivalents, are associated with the cow-calf sector (Beauchemin et al., 2010) where cattle are consuming forage-based diets often deficient in CP.

Issues with high-S diets are often considered in the feedlot industry in relation to polioencephalomalacia and adverse effects on performance (Gould, 1998; Felix et al., 2012; Drewnoski et al., 2014). In some areas of the world, high-S forages have gained attention due to issues associated with trace mineral absorption (Arthington et al., 2002). Bismuth subsalicylate (**BSS**) has been used for more than 200 years in human medicine to combat gastro-intestinal pain (Phillips et al., 2007). In the human gut, BSS binds to the S in H_2S, a known gut irritant, forming Bi_2S_3 (Suarez et al., 1998). Few experiments (Ruiz-Moreno et al., 2015) have focused on the possibility of providing BSS to reduce the negative effects of high-S on ruminant production.

It was hypothesized that providing calcium-ammonium nitrate (**CAN**) as a NPN source in combination with BSS will reduce in vitro CH_4 production and H_2S without

negatively affecting fermentation. Three experiments were designed to test this hypothesis with the following objectives: Exp. 1 – evaluate the effects of CAN on in vitro ruminal fermentation; Exp. 2 – evaluate the effects of CAN in combination with BSS on in vitro ruminal fermentation; and Exp. 3 – determine a valid inclusion rate for BSS that will reduce H_2S production without negatively affecting fermentation.

Materials and Methods

All procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee.

Experiment 1

Experimental design, substrate, and treatments

In vitro batch culture incubations were conducted as a randomized complete block design using Pensacola bahiagrass hay (*Paspalum notatum*; 80% substrate DM) and sugar cane molasses (20% substrate DM) as a basal substrate (Table 2-1). Treatments were : 1) basal substrate (**NCTRL**); 2) basal substrate with 0.75% urea (**CTRL**); 3) basal substrate with 1.2% CAN and 0.38% urea (**CAN1.2**); and 4) basal substrate with 2.4% CAN (**CAN2.4**). Treatments CTRL, CAN1.2, and CAN2.4 were isonitrogenous. The inclusion rate of CAN was selected from several previous in vivo experiments where CAN was provided to growing sheep or cattle at these concentrations (Nolan et al., 2010; van Zijderveld et al., 2011; Newbold et al., 2014). These combinations of substrate and treatments were used to evaluate CH_4 and H_2S production, NH_3-N concentration, VFA profile, and in vitro OM digestibility (**IVOMD**).

Before inclusion into the incubations, bahiagrass hay was dried for 24 h at 55°C and ground to pass a 2-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ). Reverse osmosis filtered water was used to dissolve CAN and urea, and the NPN

solutions were added to the incubations at 100 µL per 125-mL serum bottle. Bottles containing CAN1.2 received 100 µL of its corresponding CAN solution along with 100 µL of a urea solution; therefore, water was added to all bottles to avoid confounding effects of dilution or lesser headspace.

In vitro incubations

Substrates and treatments were incubated for 48 h at 39°C under constant agitation (60 rpm) in 125-mL serum bottles. Two bottles per treatment and 2 blank (without substrate or treatment) bottles were incubated in each of the 3 separate replicate days. Two ruminally-cannulated Angus-crossbred steers (348 ± 30 kg BW; average ± SD) were provided ad libitum access to bahiagrass hay and water, along with 2.27 kg of a 50:50 (as fed) molasses:glycerol mixture (5.5% CP; 75.0% TDN) for 2 weeks prior to the first ruminal fluid collection. To increase the amount of S the steers were receiving to approximately 0.35% DMI, 23.3 g of $MgSO_4$ was mixed into the liquid feed mixture daily. Steers were not adapted to CAN to eliminate any residual effects of nitrate in the inoculum of donors. A representative sample of digesta was collected and strained through 4 layers of cheese cloth, placed in a prewarmed thermos container, and transported to the laboratory within 30 min of collection. In the laboratory, ruminal fluid from the 2 steers was combined in equal proportions. A 4:1 McDougall's buffer:ruminal fluid mixture was used as inoculum for all incubations (McDougall, 1944). Bottles contained 0.7 g DM of substrate and 50 mL of inoculum. At the end of the 48 h incubations, final pH was recorded and fermentation was halted by adding 0.5 mL of a 20% H_2SO_4 solution to each bottle. Two 10-mL samples were then collected from the 125-mL serum bottles and stored at -20°C for subsequent VFA and NH_3-N analyses.

Total gas production, CH_4 and H_2S production, NH_3-N analyses

Total gas production was measured by connecting the sealed bottle to an inverted biuret filled with water allowing the headspace of the bottle to equilibrate to atmospheric pressure. The amount of water displaced by the gas was recorded as total gas production. After the pressure was released, 10 and 5 mL of gas was extracted from the sealed bottles for CH_4 and H_2S analyses, respectively.

To determine concentrations of CH_4, a gas chromatograph (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA) using flame ionization and a capillary column (Plot Fused Silica 25 m × 0.32 mm, Coating Molsieve 5A, Varian CP7536; Varian Inc., Lake Forest, CA) was used. Temperatures of the injector, column, and detector were 80, 160, and 200°C, respectively, and N_2 was the carrier gas flowing at 3.3 mL/min. The split ratio for the injected CH_4 sample was 100:1.

Production of H_2S was determined by slowly bubbling 5 mL of gas from the headspace of the serum bottles into 15-mL evacuated tubes (BD Vacutainer, Franklin Lakes, NJ) which contained 5 mL of alkaline water prepared as described by Smith et al. (2010). Tubes were vigorously shaken to allow for proper dispersion of the gas in the alkaline water. A 0.5-mL injection of *N*,*N* dimethyl-*p*-phenylenediamine sulfate was made into the tubes followed by 0.5 mL of a ferric chloride solution. Tubes were vigorously shaken again and allowed to rest for 30 min for the reaction to occur. Absorbance was read in 96-well, flat bottom plates at 665 nm using a plate reader (DU 500; Beckman Coulter Inc., Palo Alto, CA).

The phenol-hypochlorite reaction was used to determine concentrations of NH_3 as described by Broderick and Kang (1980). Samples of the post-incubation inoculum were centrifuged at 10,000 × *g* for 15 min at 4°C (Avanti J-E, Beckman Coulter Inc.).

Briefly, 1 mL of a phenol reagent was pipetted into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). A 20-µL aliquot of the supernatant from the centrifuged sample was then transferred to the phenol containing culture tubes. After vortexing, 0.8 mL of a hypochlorite solution was added to the mixture and vortexed again. The culture tubes were then covered with glass marbles and placed in a water bath at 95°C for 5 min. The only modification to the original protocol was that absorbance was read in 96-well, flat bottom plates at 665 nm using a plate reader (DU 500; Beckman Coulter Inc.).

Volatile fatty acid analysis

Concentrations of VFA were determined in a water-based solution using ethyl acetate extraction. Samples were centrifuged at 10,000 × *g* for 15 min at 4°C. Two milliliters of the supernatant was mixed with 0.4 mL (5:1 ratio) of a metaphosphoric:crotonic acid (internal standard) solution and samples were frozen overnight. Samples were then thawed and centrifuged again at 10,000 × *g* for 15 min at 4°C. Supernatant was transferred into 12 mm × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) and mixed with ethyl acetate to form a 2:1 ethyl acetate:supernatant mixture. Culture tubes were vigorously shaken and followed by a 5 min rest time to allow the separation of the ethyl acetate. A subsample of the ethyl acetate was transferred into small vials prior to analysis. Samples were analyzed with a gas chromatograph (Agilent 7820A GC, Agilent Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m × 0.53 mm, Varian CP7767; Varian Inc.). Column temperature was maintained at 110°C, and the injector and detector temperatures were 200 and 220°C, respectively.

In vitro OM digestibility

A modified Tilley and Terry (1963) procedure was used to determine IVOMD. Briefly, 0.7 g of substrate DM was incubated with 50 mL of a 4:1 McDougall's buffer:ruminal fluid inoculum in 100-mL plastic centrifuge tubes for 48 h under constant agitation (60 rpm). Two tubes per treatment and 2 blank (without substrate or treatment) tubes were incubated in each of the 3 separate replicate days. After the initial 48 h, 6 mL of HCl was added to the tubes along with 2 mL of a 5% pepsin solution. Tubes were then incubated for an additional 48 h. Samples were then filtered through P8 filters (Fisherbrand; Thermo Fisher Scientific Inc.). Filters with wet samples were then dried at 105°C in a forced air oven for 24 h to determine IVDMD. Dry samples were then placed in a muffle furnace for 6 h at 650°C. The ash was then placed in a 105°C oven for 24 h prior to recording weight.

Experiment 2

Experimental design, substrate, and treatments

In vitro batch culture incubations were conducted as a randomized complete block design with a 4 × 3 factorial arrangement of treatments. Bahiagrass hay (80% substrate DM) and sugar cane molasses (20% substrate DM) was used as the basal substrate (Table 2-1). Factors included 4 inclusion rates of BSS (0.0, 0.33, 0.66, and 1.0% substrate DM) and 3 inclusion rates of CAN (0.0, 1.2, and 2.4% substrate DM). All treatments were made isonitrogenous with urea. The inclusion rates of BSS were selected based on research performed by Ruiz-Moreno et al. (2015). The authors reported that when BSS was provided to in vitro batch and continuous cultures with a high-grain substrate, when added at up to 1.0% of the substrate DM, BSS reduced H_2S

without affecting fermentation; however, at inclusion rates greater than 1.0%, fermentation was negatively affected (Ruiz-Moreno et al., 2015).

In vitro incubations

Method of incubation was described in Exp. 1. Experiment 1 and 2 were performed simultaneously and the same ruminally-cannulated steers were used. Bismuth subsalicylate was not soluble in water; therefore, BSS was weighed into individual incubation bottles at its respective inclusion rate. All analyses performed were the same as described in Exp. 1.

Experiment 3

Experimental design, substrate, and treatment

In vitro batch culture incubations were conducted as a randomized complete block design using bahiagrass hay (79.25% substrate DM), sugar cane molasses (20% substrate DM), and urea (0.75% substrate DM) as the basal substrate (Table 2-1). Treatments included: 1) basal substrate with 0.0% BSS (**0B**); 2) basal substrate with 0.05% BSS (**5BSS**); 3) basal substrate with 0.10% BSS (**10BSS**); and 4) basal substrate with 0.33% BSS (**33BSS**). This experiment was designed to determine a more precise amount of BSS needed to have beneficial effects on ruminal fermentation using in vitro techniques. These combinations of substrate and treatments were used to evaluate CH_4 and H_2S production, VFA profile, and IVOMD, and these analyses were performed as described in Exp. 1

In vitro incubations

Method of incubation has been described in Exp. 1. Two ruminally-cannulated Angus-crossbred steers (727 ± 11 kg BW) were provided ad libitum access to bahiagrass hay and water, along with 2.27 kg of a 50:50 (as fed) molasses:glycerol

mixture (5.5% CP; 75.0% TDN) for 2 weeks prior to the first ruminal fluid collection. Steers were not supplemented with additional S. The amount of BSS that needed to be added to the serum bottles was limiting (0.00035 and 0.00231 g per serum bottle for 0.05 and 0.33%, respectively); therefore, four premixes were produced using Cr_2O_3 as an inert carrier and each premix was included in the respective serum bottles at 5 mg/bottle. All analyses performed were the same as described in Exp. 1.

Statistical Analysis

Experiment 1 was analyzed as a randomized complete block design with 3 replicated days (block) using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). For all analyses, the average of 2 tubes within day was considered the experimental unit. The following contrasts were used to aid in data interpretation: effect of NPN (NCTRL vs. others); and the linear and quadratic effects of CAN. The model included the fixed effects of treatment and the random effect of day. When random factors are included into models analyzed by SAS, SE of the means are inflated; therefore, SE of the means presented were calculated from SE of the treatment differences. Significance was declared at $P \leq 0.05$.

Experiment 2 was analyzed as a randomized complete block design with a 4 × 3 factorial arrangement of treatments using the MIXED procedure of SAS (SAS Inst. Inc.). The experimental unit was considered the mean of two tubes within day (block; n = 3/treatment). The model included the fixed effects of treatment and the random effect of day. Contrasts were used to evaluate: effect of BSS × CAN; linear, quadratic, and cubic effect of BSS; and the linear and quadratic effect of CAN. Standard error of the means presented were calculated from SE of the treatment differences. Significance was declared at $P \leq 0.05$ and Tukey-Kramer adjustments were utilized.

Experiment 3 was analyzed as a randomized complete block design with 3 replicated days (block) using the MIXED procedure of SAS (SAS Inst. Inc.). The model included the fixed effect of BSS inclusion rate and the random effect of day. Orthogonal polynomial contrasts, adjusted for unequal spacing using the IML procedure of SAS (SAS Inst. Inc.), were used to determine the linear, quadratic, and cubic effects of BSS. Standard error of the means presented were calculated from SE of the treatment differences. Significance was declared at $P \leq 0.05$ and Tukey-Kramer adjustments were used.

Results and Discussion

Experiment 1

In vitro ruminal fermentation parameters can be found on Table 2-2. There was an effect ($P = 0.0437$) of NPN on maximal gas production. When NPN was provided in the substrate, as either urea or CAN, maximal gas production was increased by 2% as compared to NCTRL; however, when CAN was provided at up to 2.4% of the substrate DM, maximal gas production was linearly reduced. The effect of NPN was expected due to the lack of available N for microbial use in the basal substrate (basal substrate CP = 7.4%). Other researchers have observed that when a source of nitrate was provided as a NPN source to ruminants consuming low quality forages, fermentation was often increased. For example, an experiment was conducted to evaluate the effects of CAN on CH_4 production and performance of Merino ewes consuming oaten chaff (CP = 4.1%) that were either defaunated or faunated (Nguyen et al., 2016). The researchers reported that when CAN was provided to the ewes, fermentation was increased leading to improvements in DM digestibility. Furthermore, CH_4 production was increased with the addition of CAN, and this was speculated to be in relation to the increase in

fermentation, and as a result, a reduction in CH_4 yield (g/kg of DMI; Nguyen et al., 2016).

By providing a source of NPN, IVOMD was improved by 10% (P = 0.0120). In accordance with the increase in maximal gas production when NPN was provided, IVOMD was likely improved because of the increase in available N for microbial growth and fermentation; however, it is evident that the source of NPN affects IVOMD. When CAN was provided at 2.4%, IVOMD was reduced (P = 0.0013) by 16% when compared to CTRL. The reduction in IVOMD with the inclusion of CAN may be partially related to a potential increase in concentration of H_2. Although, H_2 was not evaluated in this experiment, it has been reported that inclusion of nitrate can increase ruminal concentration of H_2 (Guyader et al., 2015). While this may be counter intuitive to the concept that nitrate is a H_2 sink; however, if one considers that nitrite, an intermediary of nitrate reduction to NH_3, has toxic effects on methanogens (Duin et al., 2016), it can be speculated that an increase in concentration of H_2 may be observed at times throughout fermentation. When concentration of H_2 in the rumen is elevated, the restoration of NAD^+ from NADH may be inhibited, which could lead to reduction in cellulolytic activity (Janssen, 2010).

Concentrations of NH_3-N was increased (P = 0.0010) by the addition of NPN by more than 5-fold. There was a linear (P < 0.0001) decrease of 86% when CAN was provided at up to 2.4% of the substrate DM. The reduction observed with the addition of CAN could be the result of several issues. First, the steers used in this experiment as ruminal fluid donors were not adapted to CAN. Researchers have reported the need to adapt ruminants to nitrate for at least 8 d to increase the population of nitrate and nitrite

reducers in the rumen (van Zijderveld et al., 2011; Newbold et al., 2014; Latham et al., 2016). By not adapting the donors to CAN, it is possible that the populations needed to reduce nitrate to nitrite and finally NH_3, were not available in large enough percentages to convert the CAN within 48 h. Lin et al. (2013) reported a 40% increase in NH_3-N after 24 h of in vitro incubation when the inoculum donors were adapted to nitrate rather than urea for 15 d prior to the initiation of the in vitro experiment.

All measures of CH_4 (total production m*M*, mmol/g of substrate incubated, and mmol/g of substrate fermented) were reduced linearly ($P < 0.0001$) with the addition of CAN. An 88% reduction was observed for CH_4 in mmol/g of substrate fermented when comparing CAN2.4 to CTRL. Since CAN is a NPN source, the large reduction in CH_4 production related to the addition of CAN caused the mean of NPN treatments to be lesser ($P \leq 0.0001$) than NCTRL; however, this is likely an artifact and NPN source (i.e. urea) has not been associated with a reduction in in vitro ruminal CH_4 production. Several researchers have reported in vivo and in vitro reductions of 20-30% when nitrate was included in the diet or substrate at approximately 2.0 to 2.4% of the DM (van Zijderveld et al., 2011; Newbold et al., 2014; Sun et al., 2017). The reduction in CH_4 was expected with the addition of a nitrate source; however, much of the research performed has focused on feedlot and dairy cattle consuming at least 50% concentrate. To the best of the author's knowledge, this is the first experiment evaluating the effect of CAN on the in vitro fermentation of the poor quality forage, bahiagrass.

There was no effect ($P > 0.10$) of NPN or CAN on in vitro ruminal H_2S production. Perhaps nitrate could reduce H_2S in the same manner that it reduces CH_4 production, acting as a H_2 sink; however, in this experiment, this was not observed. It may be that

the variation (SEM = 7.581) between samples was too large and masked any potential effects.

The in vitro ruminal VFA profile was not affected by NPN nor CAN ($P > 0.05$; Table 2-3). This concurs with other reports that have evaluated the addition of nitrate to forage-based substrates in vitro (Sun et al., 2017) and in vivo with a 55:45 forage to concentrate ratio (Lee et al., 2015a). It should be noted that the exact implications of nitrate on the ruminal VFA profile is still unclear. A review by Janssen (2010) provided a summary of data indicating that the VFA profile and shifts are largely related to the concentration of H_2 in the rumen. Production of VFA is shifted to acetate producing pathways, when concentration of H_2 is reduced, and to propionate producing pathways when the concentration of H_2 in the rumen is greater. It may be speculated that because the role of nitrate in the rumen is to capture H_2 that may be utilized by methanogens to produce CH_4, an increase in acetate molar proportions may be observed. Guyader et al. (2016) reported a linear increase in acetate molar proportion when nitrate was provided up to 6 m*M*.

In vitro ruminal pH was not affected by NPN nor CAN ($P > 0.10$; Table 2-2). Several other studies have reported no differences in either in vivo or in vitro ruminal pH when a source of nitrate is provided (Lund et al., 2014; Tomkins et al., 2016; Sun et al., 2017); however, other researchers have reported increased in vitro ruminal pH with an orchardgrass hay substrate (Sar et al., 2005). The similarity in pH among treatments in this experiment is possibly due to the lack of change in total concentration of VFA among treatments.

Experiment 2

No interactions ($P > 0.05$) between BSS and CAN were observed for maximal gas production, IVOMD, NH_3-N concentration, final inoculum pH, H_2S production, and the VFA profile; therefore, only the main effects of these variables will be discussed.

The main effects of BSS on in vitro ruminal fermentation parameters are presented on Table 2-4. Maximal gas production was linearly reduced ($P < 0.0001$) by 22% as BSS was included in the substrate up to 1.0% of the DM. This indicates that fermentation was negatively affected when BSS was included in the substrate DM up to 1.0%. One study using U.S. feedlot concentrate substrates, evaluated the effects of BSS on in vitro batch and continuous culture incubations (Ruiz-Moreno et al., 2015). The authors incubated up to 4% of BSS in the substrate DM in an in vitro batch culture experiment. They observed that when provided at up to 1.0% of the substrate DM, no differences occurred in maximal gas production; however, when 2 and 4% BSS was included in the DM, a 12 and 25% reduction in gas production occurred (Ruiz-Moreno et al., 2015). The difference in substrate types between the current experiment and that of Ruiz-Moreno et al. (2015) may explain why the two experiments differed in effects when BSS was provided at 1.0% of the substrate DM.

A 29% reduction (linear $P < 0.0001$) in IVOMD was observed when the inclusion rate of BSS was increased to 1.0% of the substrate DM. The linear reduction in maximal gas production when BSS was included up to 1.0% of the substrate DM followed the pattern of IVOMD. When Ruiz-Moreno et al. (2015) provided a continuous culture with 1.0% of BSS in the substrate DM, apparent and true digestibility of OM was increased. As discussed previously, the feedlot type substrate they used may have altered the effect of BSS on fermentation. It has been reported that BSS may have an impact on

certain bacteria in the human gut and feces (Manhart, 1990; Yakoob et al., 2013); thus, it may be speculated that BSS, when provided at 1.0% of the substrate DM, may alter the ruminal microbiome to have a greater ability to digest OM of a high-grain substrate compared with a forage-based substrate. The pH of the inoculum, as reported by the authors (pH = 4.8), may have had an impact on the activity of BSS in vitro (Ruiz-Moreno et al., 2015). Sox and Olson (1989) reported a large increase in the antimicrobial effects of BSS when pH was 3 compared to 7.

In vitro ruminal NH_3-N was linearly increased (P = 0.0092) as the inclusion rate of BSS increased. Concentration of NH_3-N is another indication that in vitro fermentation of bahiagrass hay and molasses was negatively affected when BSS was provided up to 1.0% of the substrate DM. The concentration of NH_3-N reported by Ruiz-Moreno et al. (2015) was essentially unchanged as BSS increased to 4.0% of the substrate DM in the batch culture and when BSS was provided to a continuous culture at 1.0% of the substrate DM. It appears that the negative effects of BSS on in vitro fermentation may be enhanced with a forage-based diet compared to a high-grain diet.

Final pH of the inoculum was increased linearly (P = 0.0004) as BSS was increased to 1.0% of the substrate DM. Similar results were reported for pH where no differences between treatments were observed until BSS was included at 2.0% of the substrate DM (Ruiz-Moreno et al., 2015). The increase in pH was more substantial with an inclusion of 4.0% BSS in the substrate DM (Ruiz-Moreno et al., 2015). All of the data related to BSS inclusion in a forage-based substrate indicate that at 1.0% of the DM, negative effects on fermentation are likely to occur.

The production of H_2S was reduced in a quadratic (P = 0.0243) manner as BSS was increased in the substrate DM to 1.0%. When included in the substrate DM at 0.33, 0.66, and 1.0%, H_2S was reduced by 61, 84, and 100%. This data is in agreement with several studies utilizing human and rat models (Suarez et al., 1998; Levitt et al., 2002; Mitsui et al., 2003). Ruiz-Moreno et al. (2015) observed similar results to those of the current experiment. When provided at 1.0% of the substrate DM, Ruiz-Moreno et al. (2015) reported a 34% reduction in H_2S production. The difference observed between the current experiment and that of Ruiz-Moreno et al. (2015) may be due to the difference in the total amount of H_2S produced between the 2 experiments. In the current experiment, substrate S concentration was 0.35% of the substrate DM and the S concentration of Ruiz-Moreno et al. (2015) was 0.44% of the substrate DM. The 25% increase in S concentration, and the reduced pH associated with high-grain diets likely increased H_2S production when compared with the current experiment. If production of H_2S was greater in the experiment reported by Ruiz-Moreno et al. (2015), then it is possible that the amount of BSS was not sufficient to reduce the H_2S at the same magnitude as the current experiment.

The main effects of CAN on in vitro ruminal fermentation parameters can be found on Table 2-5. Similar to Exp. 1, CAN linearly reduced (P = 0.0006) maximal gas production as the inclusion rate of CAN increased from 0.0 to 2.4% of the substrate DM. A 14% linear reduction (P = 0.0009) in IVOMD was observed when CAN replaced urea as an in vitro ruminal fermentation NPN source. As discussed in Exp. 1, this may be related to a shift in the concentrations of H_2 in the inoculum inhibiting cellulolytic activity (Janssen, 2010).

The concentration of NH_3-N was reduced linearly (P = 0.0001) as the inclusion rate of CAN increased to 2.4% of the substrate DM. This result mirrors what was observed in Exp. 1.

To contradict the results of Exp. 1, the production of H_2S was linearly reduced (P = 0.0113) by 66% as CAN was included in the substrate DM up to 2.4%. Although the reason for the difference in H_2S between the 2 experiments is unknown, the mode of action of nitrate may reduce H_2S production as well as CH_4. Due to the ability of nitrate to act as a H_2 sink, H_2 that may have been destined for the reduction of sulfate to H_2S, may have been captured by nitrate since the thermodynamics of nitrate reduction are more favorable than for sulfate reduction (Gibb's free energy change of -599.8 and -152.3 for nitrate and sulfate reduction, respectively; Weimer, 1998). Furthermore, it is known that nitrate and its intermediates in the pathway to NH_4 can have toxic effects on microorganisms (Zhou et al., 2012; Latham et al., 2016); therefore, if sulfate reducing bacteria are negatively impacted by supplementation of nitrate, it is probable that H_2S production would be reduced.

There was an interaction between BSS and CAN for total CH_4 production (P = 0.0097), CH_4 produced per gram of substrate incubated (P = 0.0263), and CH_4 produced per gram of substrate fermented (P = 0.0408; Table 2-6). There was an additive effect when BSS and CAN were combined in the substrate where CH_4 production was decreased in vitro. It is likely that BSS was reducing fermentation of the in vitro batch culture which led to reductions in CH_4 production. With the CH_4 being reduced due to the negative effects of BSS on fermentation, CAN continued to accept H_2 which would alternatively be used by methanogens to produce CH_4.

The effects of BSS on the in vitro ruminal VFA profile are presented on Table 2-7. The total concentrations of VFA was linearly reduced (P = 0.0025) as BSS was increased to 1.0% of the substrate DM, whereas the inclusion of CAN in the substrate DM did not impact total VFA concentration. The reduction in total concentrations of VFA aligns with the notion that BSS at 1.0% of the substrate DM negatively impacts in vitro ruminal fermentation of bahiagrass hay and molasses. When Ruiz-Moreno et al. (2015) observed negative effects on fermentation when BSS was included at 4% of the substrate DM, a reduction in the total VFA concentration was reported. Along with a reduction in total concentrations, BSS altered the molar proportion of several key VFA. A quadratic effect (P = 0.003) was observed for acetate molar proportion, where there was a reduction when BSS was included in the substrate DM up to 0.66%; however, when BSS was included at 1.0% of the substrate DM, the acetate molar proportion returned to control values. A similar, albeit opposite, quadratic (P = 0.0127) pattern occurred for propionate molar proportion. The least molar proportion of propionate was observed with 0.0% BSS, and the greatest molar proportion occurred when BSS was included in the substrate DM at 0.66%. This shift in the molar proportions of acetate and propionate produced a quadratic effect (P = 0.0049) on the A:P, with the VFA profile being more energetically efficient when BSS was included at 0.66% of the substrate DM. The shifts in molar proportions of VFA may partially be due to the concentration of H_2 concentration of the inoculum. When BSS binds to H_2S, it occurs according to the following reaction:

$$2\ Bi^{3+} + 3\ H_2S \leftrightarrow Bi_2S_3 + 6\ H^+.$$

The possibility exists that the greater concentrations of H_2 may shift VFA pathways away from the acetate producing pathways, which release H_2, making these pathways less thermodynamically favorable when concentrations of H_2 is greater, and shift them towards the energetically more efficient propionate producing pathways (Janssen, 2010). Another possibility is that BSS may be inhibitory towards bacterial populations that produce mainly acetate causing a shift in the molar proportions.

The inclusion of CAN did not affect the molar proportions of any VFA measured, nor was there any effect on the A:P ($P > 0.05$; Table 2-8). This emulates the results as discussed in Exp. 1. The pH of the in vitro ruminal batch culture inoculum after 48 h was not affected by inclusion rate of CAN ($P > 0.05$; Table 2-5).

Experiment 3

In vitro ruminal fermentation parameters of Exp. 3 are presented in Table 2-9. With the inclusion rates of BSS used in the current experiment, no differences ($P > 0.05$) were observed for IVOMD, final inoculum pH, total CH_4 production, or CH_4 produced per gram of substrate fermented. The data imply that at these inclusion rates of BSS, fermentation was not affected. Furthermore, H_2S production was linearly reduced ($P = 0.0040$) by 19% as BSS was included in the substrate DM up to 0.33%.

Interestingly, unlike Exp. 2, maximal gas production was increased in a quadratic manner ($P = 0.0050$) as BSS was increased in the substrate DM from 0.0 to 0.33%. Although there was no difference in IVOMD in the current experiment, Ruiz-Moreno et al. (2015) reported an increase in NDF, ADF, and OM digestibility of a high-concentrate substrate incubated in continuous culture for 10 d. While unknown, it may be speculated that the BSS at lesser concentrations has beneficial effects on fermentation.

There were no changes ($P > 0.05$; Table 2-10) in the VFA profile when BSS was included in the substrate DM up to 0.33%. This adds further evidence to support the idea that at lesser concentrations, BSS does not have negative effects on in vitro ruminal fermentation of bahiagrass hay and molasses.

In conclusion, the data collected from these 3 experiments indicate that CAN may be used in an effort to mitigate in vitro ruminal CH_4 production. With the inclusion rates used in these experiments, it appears that negative effects on IVOMD, concentrations of NH_3-N, and maximal gas production are disadvantage to the use of CAN to redirect metabolic H to the reduction of nitrate rather than CH_4 formation. Furthermore, it may be possible that the reduction in IVOMD, concentrations of NH_3-N, and maximal gas production related to the inclusion rate of CAN is associated with the lack of adaptation to nitrate by the ruminal fluid donors. Bismuth subsalicylate, when included in the substrate DM up to 1.0%, appears to have drastically negative effects on in vitro fermentation of bahiagrass hay and molasses. By designing another experiment to test the effects of BSS at lower inclusion rates, the author was able to provide data which implied that BSS could be included up to 0.33% of the substrate DM without reducing fermentation, while also reducing H_2S production by 19%. The combination of CAN and BSS may have beneficial impacts on the ruminal fermentation and enteric CH_4 production of ruminant animals consuming low-quality, forage-based diets.

Table 2-1. Analyzed[1] chemical composition of the basal substrate used in in vitro incubations (Exp. 1, 2, and 3)

Item	Bahiagrass hay (80% substrate DM)	Sugar cane molasses (20% substrate DM)
DM, %	90.1	81.1
OM, % DM	92.32	87.72
CP, % DM	8.1	4.5
NDF, % DM	75.7	-
ADF, % DM	42.2	-
TDN, % DM	53.0	81.0
S, % DM	0.22	0.86
Nitrate, % DM	<0.03	0.04

[1]Analyzed by a commercial laboratory using a wet chemistry package (Dairy One, Ithaca, NY).

Table 2-2. Effect of NPN and inclusion rate of CAN[1] on in vitro fermentation parameters (Exp. 1)

	Treatment[2]					*P*-value[3]		
Item	NCTRL	CTRL	CAN1.2	CAN2.4	SEM[4]	NPN	CAN-L	CAN-Q
M[5], mL	241.0	265.2	248.6	223.6	1.61	0.044	<0.001	0.077
IVOMD[6], %	38.9	46.1	43.8	38.5	0.95	0.012	0.001	0.239
NH_3-N, m*M*	0.20	2.40	1.18	0.33	0.160	0.001	<0.001	0.381
pH	6.59	6.55	6.59	6.56	0.037	0.626	0.833	0.527
Total CH_4 production, m*M*	4.48	5.00	3.08	0.40	0.144	<0.001	<0.001	0.075
CH_4, mmol/g OM incubated	1.15	1.41	0.84	0.14	0.027	<0.001	<0.001	0.139
CH_4, mmol/g OM fermented	3.00	3.09	1.92	0.37	0.120	<0.001	<0.001	0.238
H_2S, µmol/g ferm. OM	24.88	34.22	37.83	18.10	7.581	0.577	0.183	0.256

[1]CAN = Calcium ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 75% nitrate DM basis.
[2]NCTRL = 80% bahiagrass hay + 20% sugar cane molasses; CTRL = NCTRL + 0.75% urea; CAN1.2 = NCTRL + 1.2% CAN + 0.38% urea; CAN2.4 = NCTRL + 2.4% CAN (DM Basis).
[3]Observed significance levels for: NPN = NCTRL vs. the mean of CTRL, CAN1.2, and CAN2.4; CAN-L = linear effect of CAN (excludes NCTRL); CAN-Q = quadratic effect of CAN (excludes NCTRL).
[4]*n* = 3 experimental units/treatment mean.
[5]M = Maximal gas production as mL/g of OM incubated after 48 h.
[6]IVOMD = In vitro OM digestibility.

Table 2-3. Effect of NPN and inclusion of CAN[1] on in vitro ruminal VFA profile (Exp. 1)

	Treatment[2]					*P*-value[3]		
Item	NCTRL	CTRL	CAN1.2	CAN2.4	SEM[4]	NPN	CAN-L	CAN-Q
VFA, mol/100 mol								
Acetate	59.23	59.88	59.59	59.47	0.338	0.322	0.418	0.855
Propionate	31.09	30.56	30.93	30.84	0.330	0.438	0.574	0.599
Butyrate	7.82	7.71	7.69	7.89	0.115	0.653	0.326	0.449
BCVFA[5]	1.06	1.08	1.04	1.07	0.036	0.974	0.798	0.428
Valerate	0.80	0.76	0.76	0.74	0.017	0.073	0.393	0.764
Total VFA	40.86	39.96	39.51	38.67	1.136	0.303	0.453	0.896
A:P[6]	1.92	1.97	1.94	1.94	0.033	0.475	0.536	0.630

[1]CAN = Calcium ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 75% nitrate DM basis.
[2]NCTRL = 80% bahiagrass hay + 20% sugar cane molasses; CTRL = NCTRL + 0.75% urea; CAN1.2 = NCTRL + 1.2% CAN + 0.38% urea; CAN2.4 = NCTRL + 2.4% CAN (DM Basis).
[3]Observed significance levels for: NPN = NCTRL vs. the mean of CTRL, CAN1.2, and CAN2.4; CAN-L = linear effect of CAN (excludes NCTRL); CAN-Q = quadratic effect of CAN (excludes NCTRL).
[4]*n* = 3 experimental units/treatment mean.
[5]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.
[6]A:P = acetate to propionate ratio.

Table 2-4. Effect of BSS[1] on in vitro ruminal fermentation parameters (Exp. 2)

	BSS inclusion, % substrate DM					*P*-value[2]		
Item	0.0	0.33	0.66	1.0	SEM[3]	BSS-L	BSS-Q	BSS-C
M[4], mL	245.8	225.6	203.4	190.1	9.32	<0.001	0.532	0.652
IVOMD[5], %	42.8	39.6	33.1	30.4	2.03	<0.001	0.835	0.200
NH_3-N, m*M*	1.30	1.59	2.04	2.21	0.429	0.009	0.808	0.685
pH	6.56	6.63	6.70	6.78	0.054	<0.001	0.227	0.834
H_2S, µmol/g ferm. OM	30.05	11.66	4.68	0.00	4.910	<0.001	0.024	0.480

[1]BSS = bismuth subsalicylate.
[2]Observed significance levels for: BSS-L = linear effect of BSS; BSS-Q = quadratic effect of BSS; BSS-C = cubic effect of BSS.
[3]n = 9 experimental units/treatment mean.
[4]M = Maximal gas production as mL/g of OM incubated after 48 h.
[5]IVOMD = In vitro OM digestibility.

Table 2-5. Effect of CAN[1] on in vitro ruminal fermentation parameters (Exp. 2)

	CAN inclusion, % substrate DM				*P*-value[2]	
Item	0.0	1.2	2.4	SEM[3]	CAN-L	CAN-Q
M[4], mL	228.8	217.6	202.3	9.32	<0.001	0.721
IVOMD[5], %	38.9	37.2	33.3	2.03	<0.001	0.372
NH_3-N, m*M*	2.59	1.59	1.17	0.429	<0.001	0.287
pH	6.63	6.68	6.70	0.054	0.099	0.657
H_2S, µmol/g ferm. OM	14.56	15.27	4.96	4.910	0.011	0.080

[1]CAN = Calcium ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 75% nitrate DM basis.
[2]Observed significance levels for: NPN = NCTRL vs. the mean of CTRL, CAN1.2, and CAN2.4; CAN-L = linear effect of CAN (excludes NCTRL); CAN-Q = quadratic effect of CAN (excludes NCTRL).
[3]*n* = 9 experimental units/treatment mean.
[4]M = Maximal gas production as mL/g of OM incubated after 48 h.
[5]IVOMD = In vitro OM digestibility.

Table 2-6. Effect of BSS[1] and CAN[2] on in vitro ruminal CH_4 production (Exp. 2)

	BSS, % substrate DM															
	0			0.33			0.66			1.0						
	CAN, % substrate DM														*P*-value[3]	
Item	0	1.2	2.4	0	1.2	2.4	0	1.2	2.4	0	1.2	2.4	SEM[4]	BSS	CAN	B×N
CH_4, m*M*[5]	5.00	3.08	0.40	3.89	1.10	0.00	2.06	0.00	0.00	0.38	0.00	0.00	0.524	<0.01	<0.01	<0.01
CH_4, inc[6]	1.41	0.84	0.14	1.07	0.35	0.00	0.58	0.00	0.00	0.09	0.00	0.00	0.160	<0.01	<0.01	0.026
CH_4, ferm[7]	3.09	1.92	0.37	2.44	0.85	0.00	1.36	0.00	0.00	0.27	0.00	0.00	0.365	<0.01	<0.01	0.041

[1]BSS = bismuth subsalicylate.
[2]CAN = Calcium ammonium nitrate (5Ca(NO3)2-NH4NO3) – 75% nitrate DM basis.
[3]Observed significance levels for: BSS = main effect of BSS; CAN = main effect of CAN; B×N = interaction of BSS and CAN.
[4]n = 3 experimental units/treatment mean.
[5]CH_4, m*M* = total CH_4 production after 48 h (m*M*).
[6]CH_4, inc = mmol of CH_4 produced per gram of OM incubated.
[7]CH_4, ferm = mmol of CH_4 produced per gram of OM fermented.

Table 2-7. Effect of BSS[1] on in vitro ruminal VFA profile (Exp. 2)

	BSS inclusion, % substrate DM					*P*-value[2]		
Item	0.0	0.33	0.66	1.0	SEM[3]	BSS-L	BSS-Q	BSS-C
VFA, mol/100 mol								
Acetate	59.65	58.62	58.35	59.08	0.351	0.041	0.003	0.771
Propionate	30.78	31.80	32.45	31.90	0.501	0.005	0.013	0.528
Butyrate	7.76	7.93	7.70	7.49	0.228	0.092	0.173	0.484
BCVFA[4]	1.06	0.95	0.82	0.85	0.132	0.034	0.367	0.631
Valerate	0.76	0.71	0.69	0.68	0.020	<0.001	0.086	0.682
Total VFA	39.38	36.47	35.49	35.23	1.520	0.003	0.146	0.762
A:P[5]	1.95	1.86	1.81	1.86	0.041	0.007	0.005	0.558

[1]BSS = bismuth subsalicylate.
[2]Observed significance levels for: BSS-L = linear effect of BSS; BSS-Q = quadratic effect of BSS; BSS-C = cubic effect of BSS.
[3]*n* = 9 experimental units/treatment mean.
[4]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.
[5]A:P = acetate to propionate ratio.

Table 2-8. Effect of CAN[1] on in vitro ruminal VFA profile (Exp. 2)

	CAN inclusion, % substrate DM				*P*-value[2]	
Item	0.0	1.2	2.4	SEM[3]	CAN-L	CAN-Q
VFA, mol/100 mol						
Acetate	59.04	58.96	58.78	0.351	0.307	0.787
Propionate	31.60	31.62	31.97	0.501	0.302	0.595
Butyrate	7.71	7.78	7.68	0.228	0.877	0.610
BCVFA[4]	0.94	0.94	0.87	0.132	0.485	0.640
Valerate	0.72	0.70	0.70	0.020	0.117	0.773
Total VFA	37.24	37.02	35.68	1.520	0.160	0.554
A:P[5]	1.88	1.88	1.85	0.041	0.300	0.724

[1]CAN = Calcium ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 75% nitrate DM basis.
[2]Observed significance levels for: CAN-L = linear effect of CAN; CAN-Q = quadratic effect of CAN.
[3]n = 9 experimental units/treatment mean.
[4]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.
[5]A:P = acetate to propionate ratio.

Table 2-9. Effect of increasing inclusion rate of BSS[1] on in vitro ruminal fermentation parameters (Exp. 3)

	BSS inclusion, % substrate DM					*P*-value[2]		
Item	0.0	0.05	0.10	0.33	SEM[3]	Linear	Quad.	Cubic
M[4], mL	277.9	284.7	286.8	289.0	1.11	<0.001	0.005	0.256
IVOMD[5], %	53.6	53.3	53.7	55.4	0.86	0.114	0.631	0.825
pH	6.50	6.49	6.49	6.48	0.007	0.099	0.607	0.334
Total CH_4 production, m*M*	5.58	5.71	5.69	5.70	0.064	0.461	0.296	0.433
CH_4, mmol/g OM incubated	1.68	1.77	1.78	1.79	0.020	0.029	0.034	0.275
CH_4, mmol/g OM fermented	2.89	3.05	3.04	2.97	0.070	0.899	0.144	0.444
H_2S, µmol/g ferm. OM	25.63	25.31	25.27	20.69	0.881	0.004	0.355	0.822

[1]BSS = bismuth subsalicylate.
[2]Observed significance levels for: Linear = linear effect of BSS; Quad. = quadratic effect of BSS; Cubic = cubic effect of BSS.
[3]*n* = 3 experimental units/treatment mean.
[4]M = Maximal gas production as mL/g of OM incubated after 48 h.
[5]IVOMD = In vitro OM digestibility.

Table 2-10. Effect of increasing inclusion rate of BSS[1] on in vitro ruminal VFA profile (Exp. 3)

	BSS inclusion, % substrate DM					*P*-value[2]		
Item	0.0	0.05	0.10	0.33	SEM[3]	Linear	Quad.	Cubic
VFA, mol/100 mol								
Acetate	64.59	64.56	65.87	67.27	1.326	0.159	0.852	0.678
Propionate	23.45	23.52	21.98	20.44	1.454	0.149	0.839	0.649
Butyrate	10.06	10.05	10.08	10.01	0.071	0.627	0.806	0.794
BCVFA[4]	0.90	0.90	1.08	1.26	0.178	0.162	0.837	0.670
Valerate	0.99	0.98	1.00	1.02	0.022	0.228	0.960	0.494
Total VFA	43.06	44.81	38.25	32.05	5.281	0.130	0.932	0.536
A:P[5]	2.76	2.75	3.10	3.46	0.339	0.151	0.841	0.663

[1]BSS = bismuth subsalicylate.
[2]Observed significance levels for: Linear = linear effect of BSS; Quad. = quadratic effect of BSS; Cubic = cubic effect of BSS.
[3]*n* = 3 experimental units/treatment mean.
[4]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.
[5]A:P = acetate to propionate ratio.

CHAPTER 3
EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON RUMINAL FERMENTATION OF BEEF CATTLE

Introduction

It is widely accepted that the addition of nitrate to ruminants can mitigate enteric CH_4 production (van Zijderveld et al., 2010; Newbold et al., 2014; Olijhoek et al., 2016). The reduction in CH_4 is due to nitrate acting as a H_2 sink in the rumen and having negative effects on methanogenic archaea (Zhou et al., 2012; Duin et al., 2016). Researchers have evaluated the impacts of nitrate supplementation on ruminal fermentation, but a majority of the research has focused on cattle consuming at least 30% concentrate.

It has been speculated that nitrate could be substituted for urea as a NPN source while mitigating enteric CH_4 production (Leng, 2008). Researchers have evaluated microbial CP synthesis in vivo and in vitro with differing results (Li et al., 2012; Li et al., 2013; Guyader et al., 2016); however, there has not been a focus on cattle consuming low-quality, forage-based diets, in which case NPN would be beneficial.

Bismuth subsalicylate (**BSS**) is not a novel compound in human health; however, very little research has evaluated its effects in ruminants (Ruiz-Moreno et al., 2015). In humans, BSS has been reported to decrease H_2S in the gut by binding the sulfide (Suarez et al., 1998), and to have antimicrobial effects (Manhart, 1990; Bland et al., 2004; Yakoob et al., 2013). In in vitro ruminal fermentation, BSS has been reported to reduce H_2S and, while at lesser inclusion rates, have minimal effects on fermentation (Ruiz-Moreno et al., 2015).

It was hypothesized that the addition of encapsulated calcium-ammonium nitrate (**eCAN**) to cattle consuming a bahiagrass hay-based diet will shift ruminal fermentation

to acetate producing pathways, and have similar microbial CP synthesis compared with urea. Providing BSS was hypothesized to reduce H_2S concentration in the ruminal gas cap while not shifting ruminal fermentation. The objective of this experiment was to evaluate the effects of the combination of eCAN and BSS on ruminal fermentation and microbial protein synthesis.

Materials and Methods

All procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental Design, Animals, and Treatments

The experiment was conducted at the University of Florida – North Florida Research and Education Center Beef Unit (**UF – NFREC BU**) in Marianna, FL. Ten ruminally cannulated Angus-crossbred steers (n = 8; 461 ± 148 kg of BW; average BW ± SD) and heifers (n = 2; 337 ± 74 kg of BW) were used in a duplicated 5 × 5 Latin square design with a 2 × 2 + 1 factorial arrangement of treatments. In each of the five 28-d periods, d 0 to 13 were for adaptation to the diet and treatments; on d 0, 1, 2, 3, and 14 ruminal gas cap samples were collected; from d 13 to 17, the cattle were brought into the UF – NFREC BU Pavilion to collect data for DMI, blood and ruminal parameters, and omasal spot samples; on d 21 and 22, ruminal evacuations were performed and the cattle were dosed with Cr-EDTA and $YbCL_3$ for digesta flow rate analysis; and d 23 to 28 were for washout, when all the cattle only received bahiagrass hay and sugar cane molasses. At the beginning of the first period, all cattle were randomly assigned to 1 of 5 treatments: 1) **NCTRL**, no added NPN or BSS; 2) **U**, urea supplemented at 182 mg/kg of BW; 3) **NIT**, nitrate, in the form of eCAN, supplemented at 350 mg/kg of BW; 4) **UB**, urea supplemented at 182 mg/kg of BW and BSS

supplemented at 58.4 mg/kg of BW; and 5) **NITB**, nitrate, in the form of eCAN, supplemented at 350 mg/kg of BW and BSS supplemented at 58.4 mg/kg of BW. Treatments U, NIT, UB, and NITB were isonitrogenous.

Throughout the 5 periods, the cattle were kept in individual pastures (0.69 ha; d 0 to 13, d 17 to 21, and d 23 to 28) and pens in the UF – NFREC Beef Unit Pavillion (13.4 m^2; d 13 to 17 and d 21 to 22). The cattle had ad libitum access to Pensacola bahiagrass (*Paspalum notatum*) hay and were supplemented daily with molasses at 2.7 g/kg of BW. When on pasture, cattle received full length bahiagrass hay as round bales; however, to be able to provide the same source of hay fed to cattle on dormant pastures, when the cattle were in the Pavilion, bahiagrass hay was chopped using a Tub Grinder (Haybuster, Jamestown, ND) and square baled. Round bales of hay were sampled at the beginning of each period for nutrient analysis using a hand drill and core sampler and square bales were sampled by hand as the hay was fed to the cattle. The amount of molasses corresponding to each animal was weighed and offered daily using rubber containers inside pastures and pens. The sugar cane molasses was provided by Quality Liquid Feed (Dodgeville, WI). Chemical composition of bahiagrass hay and sugar cane molasses while on pasture and while confined in the UF – NFREC BU Pavilion are presented on Table 3-1.

To reduce any negative effects of nitrate on the cattle, on d 0 cattle began an adaptation to eCAN and urea. On d 0 and 1, cattle received 20% of their total supplemental N; on d 2 and 3, cattle received 40% of their total supplemental N; on d 4 and 5, cattle received 60% of their total supplemental N; on d 6 and 7, cattle received

80% of their total supplemental N; and beginning on d 8, cattle were receiving 100% of their total supplemental N as urea or eCAN.

Sampling Procedures

All protocols and procedures used for collecting samples and data from animals were used in an identical manner throughout all 5 periods.

Ruminal gas cap sampling

On d 0, ruminal gas cap samples were collected at 0, 3, 6, 9, and 12 h post feeding of molasses. On d 1, 2, 3, and 14, samples were collected at h 0, just prior to feeding molasses. To collect the sample, a 10-mL syringe fitted with a one-way valve and 16-gauge needle was inserted through the cannula plug to prevent as much contamination with environmental air as possible. Once the needle was inside the rumen, the plunger of the syringe was used 5 times to thoroughly mix the gas sample surrounding the needle. Then 5 mL of gas cap were drawn into the syringe, at which point, the one-way valve was closed to prevent contamination. The sample was then injected into a 10-mL evacuated tube (BD Vacutainer, Franklin Lakes, NJ) containing 5 mL of alkaline water (pH 8.5 to 9). The samples were transported to the laboratory and immediately analyzed for H_2S concentration.

Ruminal fluid, pH, blood sampling, and rectal temperature

Ruminal fluid and blood were collected, and rectal temperature and pH were recorded over a 72 h period. Briefly, on d 14 of each period samples were collected at 0800, 1400, and 2000 h; on d 15 at 0200, 1000, 1600, and 2200 h; on d 16 at 0400, 1200, and 1800 h; and on d 17 at 0000 and 0600h. A representative sample of ruminal digesta was collected and strained through 4 layers of cheesecloth, and pH was immediately measured using a manual pH meter (Corning Pinnacle M530), Corning

Inc., Corning, NY). A 10-mL sample of the ruminal fluid was taken into a 15-mL conical tube and mixed with 0.1 mL of a 20% (vol/vol) H_2SO_4 to halt fermentation and preserve NH_3-N and VFA. Samples of ruminal fluid were stored at -20°C for further analysis.

Blood samples were collected via jugular venipuncture into 10-mL evacuated tubes which contained Na heparin (BD Vacutainer), placed on ice, and centrifuged at 1,500 × *g* for 15 min at 4°C. Plasma was then transferred to polypropylene vials (12 × 75 mm; Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) and stored at -20°C for further analysis.

Hay and total dry matter intake

On d 13 of each period, the cattle were moved from their respective pastures into the UF – NFREC BU Pavilion. From d 13 to 17, hay DMI was measured by weighing bahiagrass hay (as is) prior to feeding and then weighing orts after 24 h. Samples of hay and orts were collected each d for chemical analysis. The cattle consumed all of the molasses provided to them while DMI was being measured; therefore, there were no molasses orts to be weighed.

Hay, molasses, and orts were also sampled on d 21 and 22 of each period to determine DM, OM, and indigestible NDF (**iNDF**) entering the rumen. These values were used for digesta flow calculations.

Omasal sampling

Omasal spot sampling occurred on d 14 to 17 at the same 12 time points as described in the **Ruminal fluid, pH, blood sampling, and rectal temperature** subsection. Omasal samples were collected with a device constructed as follows: first, a vacuum pump was connected using a rubber hose and rubber stopper to a 500-mL polypropylene side arm flask; the first side arm flask was connected to a second

identical flask using a rubber hose, connecting the two side arms. The collection hose, a 1.9 cm (inside diameter) clear rubber hose with fiber reinforcement, was fitted to the top of the second side arm flask using a rubber stopper; a 50-mL scintillation vial was filled with ball bearings, sealed closed, and attached to the end of the collection hose to act as a weight aiding in the placement of the hose, without hindering flow.

To collect the sample, the omasal orifice was located and the weight at the end of the collection hose was inserted into the orifice. The hose was then placed further into the omasum to attempt to collect strictly omasal samples; furthermore, the first 250-mL sample collected was discarded in case ruminal fluid had entered the collection tube.

Samples were collected into the second side arm flask until the volume reached 250 mL. The 250-mL sample was then transferred into a plastic freezer storage bag and placed on ice until transported to the laboratory. Omasal samples were stored at -20°C for further analysis.

To separate the fractions of the omasal samples, equal volumes (150 mL) of each individual time point was combined to create one composite representative of 1 animal for 24 h post feeding in 1 period. The composites were filtered through 1 layer of cheesecloth and the residue left in the cheesecloth was considered the large particle fraction. The filtrate was centrifuged at 1,000 × *g* for 5 min at 4°C and the supernatant was considered the liquid fraction and the pellet was considered the small particle fraction. Both solid fractions were weighed wet and subsamples were taken for DM, OM, iNDF, N, and purines analysis. Volume and weight were recorded of the liquid fraction and a sub-sample was collected for DM, OM, N, and purine analysis.

Reconstruction of omasal composites, in DM, was calculated from the DM of the differing fractions.

Bacterial pellet for purine reference

On d 15 at 1000, 1600, and 2200 h, a representative sample of whole rumen content was collected to isolate particle associate bacteria and liquid associated bacteria according to Martínez et al. (2009). The digesta was collected and placed into plastic freezer storage bags and placed on ice until transported to the laboratory. Once in the laboratory, samples were strained through 4 layers of cheesecloth to separate the liquid and solid fractions. The liquid fraction was then centrifuged at 1000 × *g* for 5 min. The supernatant was collected and centrifuged at 25,000 × *g*. The supernatant was discarded and the liquid associated bacterial pellet was stored at -20°C for further analysis. To isolate the particle associated bacteria from the ruminal solids, a 50 g (as is) subsample was incubated at 39°C under constant agitation (60 rpm) for 15 min with 150 mL of a saline solution (0.9% NaCl) which contained 0.1% methylcellulose. After incubation, 25 mL of cold saline-methylcellulose solution (4°C) was added and the solids were stored at 4°C for 24 h. Once removed from the 4°C environment, the solids were placed in a blender (Waring Products Division, New Hartford, CT) and homogenized for 10 s. The resulting solution was filtered through two layers of nylon cloth (50-µm pore size) and treated in an identical manner to the liquid fraction. The particle associated bacterial pellet was stored at -20°C for further analysis.

Ruminal evacuations and digesta flow marker dosing

On d 21, the cattle were brought into the UF – NFREC BU Pavilion at 0900 h (1 h post feeding of molasses). The cattle were immediately brought through the chute for ruminal evacuation. Ruminal contents were evacuated manually into large containers so

that weight and volume of the ruminal contents could be recorded. When the dimensions of the ruminal contents were recorded, the digesta was returned to the respective rumen. An aliquot representing 10% of the total digesta was separated and stored on ice until transported to the laboratory. Ruminal aliquots were stored at -20°C for further analysis.

To determine the ruminal nutrient pool sizes, aliquots were thawed and fractionated into 3 phases: liquid fraction, large particles, and small particles. Briefly, the whole digesta sample (representing 10% of the total ruminal digesta) was strained through 1 layer of cheesecloth. The content remaining within the cheesecloth was considered the large particle phase. The filtrate was then centrifuged at 1000 × g for 5 min at 4°C. The supernatant was considered the liquid fraction phase and the pellet was considered the small particle phase. All 3 of the phases were weighed and sub samples were taken and lyophilized for further analysis. Ruminal pool sizes (kg) of DM and OM were determined by multiplying the concentration of each component by the DM mass of each of the 3 phases.

Chromium-EDTA and $YbCl_3$, external and inert markers, were used to mark the liquid fraction and small particle phases of the digesta, respectively. Once the weight and volume of ruminal contents had been recorded and contents returned to their respective rumen, a 1-L Cr-EDTA and $YbCl_3$ solution [2.77 g of Cr and 2.2 g Yb; modified from Binnerts et al. (1968)] was poured into the rumen using a funnel and rubber hose (1 m). Ruminal contents were mixed for 3 min to aid in equilibration of the markers in the rumen. Whole ruminal contents were collected every 3 h post dosing for 21 h and strained through 1 layer of cheesecloth to determine a ruminal liquid and small

particle dilution rates (Romero et al., 2013). Indigestible NDF was determined in the large and small particle phases (not in the liquid fraction phase; Ahvenjärvi et al., 2000).

Laboratory Analyses

All protocols and procedures used for analyzing samples and data from animals were used in an identical manner throughout all 5 periods.

Hydrogen sulfide concentration

Concentration of H_2S in the ruminal gas cap was determined as described by Henry et al. (2015). Briefly, A 0.5-mL injection of *N*,*N* dimethyl-*p*-phenylenediamine sulfate was made into the tubes followed by 0.5 mL of a ferric chloride solution. Tubes were shaken vigorously and allowed to rest for 30 min for the reaction to occur (Smith et al., 2010). Absorbance was read in 96-well, flat bottom plates at 665 nm using a plate reader (DU 500; Beckman Coulter Inc., Palo Alto, CA).

Volatile fatty acid profile

A water-based solution using ethyl acetate extraction was used to determine VFA concentrations in the ruminal fluid samples. Samples were centrifuged at 10,000 × *g* for 15 min at 4°C. Two milliliters of the supernatant was mixed with 0.4 mL (5:1 ratio) of a metaphosphoric:crotonic acid (internal standard) solution and samples were frozen overnight. Samples were then thawed and centrifuged again at 10,000 × *g* for 15 min at 4°C. Supernatant was transferred into 12 mm × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) and mixed with ethyl acetate to form a 2:1 ethyl acetate:supernatant mixture. Culture tubes were vigorously shaken and followed by a 5 min rest time to allow the separation of the ethyl acetate. A subsample of the ethyl acetate was transferred into small vials prior to analysis. Samples were analyzed with a gas chromatograph (Agilent 7820A GC, Agilent

Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m × 0.53 mm, Varian CP7767; Varian Inc.). Column temperature was maintained at 110°C, and the injector and detector temperatures were 200 and 220°C, respectively.

Ammonia-N and blood urea N concentration

The phenol-hypochlorite reaction was used to determine NH_3-N concentration as described by Broderick and Kang (1980). Ruminal fluid samples were centrifuged at 10,000 × *g* for 15 min at 4°C (Avanti J-E, Beckman Coulter Inc.). Briefly, 1 mL of a phenol reagent was pipetted into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). A 20-µL aliquot of the supernatant from the centrifuged ruminal fluid was then transferred to the phenol containing culture tubes. After vortexing, 0.8 mL of a hypochlorite solution was added to the mixture and vortexed again. The culture tubes were then covered with glass marbles and placed in a water bath at 95°C for 5 min. The only modification to the original protocol was that absorbance was read in 96-well, flat bottom plates at 665 nm using a plate reader (DU 500; Beckman Coulter Inc.).

Plasma was analyzed for blood urea N (**BUN**) using a quantitative colorimetric kit (B7551-120; Pointe Scientific Inc., Canton, MI).

Chemical analyses of hay, orts, ruminal, and omasal digesta fractions

Hay and orts were analyzed for DM and OM on all d of collection (d 13 to 17 and d 21 and 22). On d 21 and 22, hay and orts were analyzed for iNDF.

To determine DM of hay and orts, samples were weighed prior to being placed in a 55°C forced air oven for 72 h. Dry, hot weight was used to calculate DM of the sample. The sample was then ground to pass through a 2-mm screen in a Wiley mill

(Thomas Scientific, Swedesboro, NJ). To determine OM, 0.5 g of ground sample (in duplicate) was weighed into ceramic crucibles and placed in a 105°C forced air oven for 24 h to determine sample DM. Dried samples were then placed in a 650°C muffle furnace for 6 h before returning to a 105°C forced air oven. Hot, ashed samples were weighed and used to calculate OM.

To determine DM of ruminal and omasal fractions, samples were weighed wet prior to lyophilization. After being lyophilized, samples were placed in a 55°C oven for 24 h prior to being weighed. To determine OM, the same procedure as described for hay and orts was used.

The concentration of iNDF in the hay, orts, ruminal, and omasal solid fractions was determined as described by Gregorini et al. (2008), Cole et al. (2011), and Krizsan and Huhtanen (2013). Briefly, 0.5 g of sample was weighed into Ankom F57 filter bags (Ankom Technology Corp. Macedon, NY) and then incubated into the rumen of a cannulated steer grazing a bahiagrass and bermudagrass (*Cynadon dactylon*) mixed pasture for 288 h to ensure complete digestion of potentially digestible NDF. After incubation samples were rinsed 2 times with warm tap water followed by 4 rinses with water filtered through a reverse osmosis system. The rinsed samples were then analyzed for NDF, using heat-stable α-amylase and sodium sulfite as described by Van Soest et al. (1991) using an Ankom 200 Fiber Analyzer (Ankom Technology Corp.).

Ruminal and omasal fractions were analyzed for total N using a CHNS analyzer by the Dumas dry combustion method (Vario Micro Cube; Elementar, Hanau, Germany). Crude protein was calculated by multiplying the N concentration of the dry sample by 6.25.

Chromium concentrations

For concentrations of Cr, ruminal fluid samples were centrifuged at 1,000 × *g* at 4°C for 5 min. The supernatant was transferred to 15-mL conical tubes and the small particle pellet was lyophilized for further analysis. Atomic absorption spectrophotometry (359.4 nm with an air-plus-acetylene flame; AAnalyst 200; PerkinElmer, Walther, MA) was used to determine the concentration of Cr in the ruminal liquid fraction after dilution with deionized water. The natural logarithm of Cr concentration in the small liquid fraction was regressed against time to calculate liquid fraction flow rate (Romero et al., 2013).

Purine analysis

The purine content of omasal digesta and ruminal bacteria (liquid and particle associated) was determined as described by Zinn and Owens (1986) using Torula Yeast RNA as a standard. Briefly, 0.5 g of omasal sample and 0.2 g of bacterial sample were hydrolyzed by incubating the samples in 25-mL borosilicate screw cap borosilicate culture tubes (Fisherbrand; Thermo Fisher Scientific Inc.) with 2.5 mL of a 70% perchloric acid in a 95°C water bath. After 15 min, the samples were vortexed and returned to the water bath for an additional 45 min. A dilute buffer (0.0285 *M* $NH_4H_2PO_4$) was added to the solution at 17.5 mL before vortexing. The tubes were then returned to the water bath for 15 min prior to filtration (Whatman #541; GE Healthcare UK Limited, Buckinghamshire, UK). To determine the purine content, 0.5 mL of the filtrate was transferred into 50-mL round-bottom centrifuge tubes followed by 0.5 mL of 0.4 *M* $AgNO_3$ and 9 mL of a 0.2 *M* $NH_4H_2PO_4$ buffer. The samples were then stored at 4°C for 12 h before centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was removed and discarded. Without disturbing the pellet, 10 mL of acidic reverse-osmosis

filtered water (made pH 2 using H_2SO_4) was used to wash the pellet. The tubes were centrifuged again at 10,000 × *g* for 10 min at 4°C and the supernatant was removed and discarded. A 0.5 *N* HCl solution was added at 10 mL and mixed using a pasteur pipette. The tube was then incubated in a 95°C water bath for 30 min. The samples were vortexed and centrifuged at 10,000 × *g* for 10 min at 4°C. The absorbance of the supernatant was then read at 260 nm (ultra-violate) using a spectrophotometer (DU-530, Beckman Coulter, Palo Alto, CA).

Calculations

Flow of digesta

Large particle digesta flow was calculated as described by Linneen et al. (2015):

Large particle digesta flow (%/h) = iNDF intake (g/h) ÷ [d 21 ruminal large particle iNDF (kg) + d 22 ruminal large particle iNDF (kg)] ÷ 2.

The flow rate of the liquid fraction was calculated by regressing the natural logarithm of the marker concentration against time. The absolute value of the slope was considered flow rate in percent of volume per h.

All fractional rates of passage were then multiplied by the mean ruminal DM mass of their respective fraction to calculate grams per hour and, further, kilograms per 24 h.

Flow and efficiency of microbial N

Microbial N flow to the omasum was calculated using liquid associated bacteria and particle associated bacteria as references and total purines as microbial markers as follows:

microbial N flow (g/d) = digesta flow (kg/d) × purines in digesta (μmol/g) × g of N/μmol of purines in bacterial reference.

The true digestibility of OM in the rumen was calculated as described by Gozho et al. (2009):

OM truly digested (% of total OM) = OM intake (kg/d) – {[OM flowing to the omasum (kg/d) –bacterial OM flowing to the omasum (kg/d)] ÷ OMI kg/d} × 100

Microbial N efficiency was calculated as described by Gozho et al. (2009):

Microbial N efficiency = microbial N flowing to the omasum (g/24 h) ÷ OM truly digested (kg/24 h).

Statistical Analysis

Data were analyzed as a duplicated 5 × 5 Latin square with repeated measures for blood and ruminal fermentation parameters (H_2S on d 0, BUN, NH_3-N, ruminal pH, and VFA) using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). For hay and total DMI, H_2S on d 1, 2, and 3, digesta flow, and microbial N efficiency, the model included the fixed effects of treatment, square, period, and animal within square. For parameters analyzed using repeated measures, the model included the fixed effects of treatment, square, period, time, treatment × time, and animal within square. The random effect of animal within treatment was used to designate the denominator degrees of freedom. Animal within period was considered the subject and the covariance structure used for all parameters was compound symmetry based on the lesser Akaike information criterion value. To aid in the interpretation of data, the following contrasts were used: the effect of NPN = NCTRL vs. the mean of U, NIT, UB, and NITB; the effect of NPN source = the mean of U and UB vs. the mean of NIT and NITB; the effect of BSS = the mean of U and NIT vs. the mean of UB and NITB; and NPN source × BSS = the mean of U and NITB vs. the mean of NIT and UB. Significance was declared at $P \leq 0.05$.

Results and Discussion

Nitrate has been evaluated extensively in the past in ruminants consuming diets that range from 50 to 90% concentrate (Olijhoek et al., 2016; Lee et al., 2017a; Lee et al., 2017b). The vast majority of this research has focused on mitigating enteric CH_4 emissions and performance of cattle (beef and dairy) and sheep (van Zijderveld et al., 2010; van Zijderveld et al., 2011; Newbold et al., 2014). There is a lack of data evaluating the effects of nitrate on forage fed animals, which produce nearly three times as much CH_4 as grain-fed (Henry et al., 2015). The current experiment utilized bahiagrass hay and molasses, a common wintering diet for beef cattle in the southeast U. S.

Cattle consuming forage-based diets, such as the current experiment, rarely have issues with S-induced polioencephalomalacia (Gould, 1998) because the ruminal pH is not optimal for the undissociated sulfide (pK_a 7.04); however, other negative effects of S have been reported, such as impacts on trace mineral absorption (Arthington et al., 2002; Pogge et al., 2014).

Ruminal gas cap H_2S concentration and rectal temperature data can be found on Table 3-2. There was no treatment × time interaction (P = 0.7880) for ruminal H_2S concentration, nor was there an effect (P > 0.05) of NPN, NPN source, or BSS. A decrease in H_2S concentration was observed for animals consuming NPN, regardless of source, on d 3 (P = 0.0179). Neither NPN source nor BSS impacted H_2S concentration of the ruminal gas cap on d 1, 2, 3 or 14 (P > 0.05). It was hypothesized that nitrate, which reduces CH_4 via two methods – acting as a H_2 sink (Ungerfeld and Kohn, 2006) and toxic effects on methanogens (Zhou et al., 2012; Duin et al., 2016), could have similar effects on H_2S production in the rumen as a H_2 sink. Bismuth subsalicylate was

also expected to reduce concentrations of H_2S in the rumen. Data from a series of in vitro experiments presented and discussed in Chapter 2 indicated that BSS was a strong inhibitor of H_2S. When provided at 0.33% of a bahiagrass hay and molasses substrate (DM basis), BSS reduced production of H_2S by 61%. Furthermore, research evaluating BSS in a high-grain substrate observed a 34% reduction in production of H_2S (Ruiz-Moreno et al., 2015). In the current experiment, concentrations of H_2S were analyzed prior to feeding molasses on d 1, 2, 3, and 14. It is probable that the effects of treatment were no longer observable. The fermentation of molasses, where the majority of the dietary S is found, is rapid, and ruminal pH reached the nadir 2 h post-feeding of molasses (Figure 3 - 1). Future studies evaluating the effects of nitrate and BSS on H_2S production should consider evaluating ruminal concentrations 2 to 4 h post-feeding.

There was no treatment × time interaction (P = 0.1795) for rectal temperature. Rectal temperature was evaluated in this experiment because previous work in humans has indicated that nitrates lead to vasodilation (Butler and Feelisch, 2008). The author speculated that if providing nitrate caused vasodilation of cattle, perhaps body temperature might be lessened. That hypothesis was not supported by the data in this experiment, as the addition of eCAN, in place of urea, in the diet of cannulated cattle did not affect (P = 0.2910) rectal temperature. Interestingly, a decrease (P = 0.0087) in rectal temperature was observed when cattle were provided BSS. A vast majority of research focusing on bismuth compounds (i.e., BSS) has been performed in human and rat models (Suarez et al., 1998; Levitt et al., 2002). To the best of the author's knowledge, this experiment represents the first data representing the effects of BSS in ruminants (in vivo). It may be possible that as bismuth can act as a nonsteroidal

antiinflammatory drug (NSAID) in humans (Hawksworth et al., 2014), that BSS may have similar effects in cattle. Furthermore, salicylate can alter body temperature by inhibiting both COX-1 and COX-2. Through these inhibitions, prostaglandins, which cause inflammation and increased body temperature, are reduced (Cashman, 1996).

Data related to DMI, ruminal pH, NH_3-N and BUN concentration is presented on Table 3-3. There were no effects observed on bahiagrass hay or total DMI ($P > 0.05$).

There was a treatment × time interaction for ruminal pH ($P = 0.0002$; Figure 3-1), ruminal concentration of NH_3-N ($P < 0.0001$; Figure 3-2), and BUN ($P < 0.0001$; Figure 3-1). For ruminal pH, NCTRL exhibited a lesser pH compared with U, NIT, and UB 2 h postfeeding of molasses ($P < 0.03$). Cattle consuming NCTRL also had lesser ruminal pH at 6 h postfeeding when compared with U and UB ($P < 0.04$). Likely NCTRL had a more acidic ruminal environment because of the lack of NPN source to be converted to NH_3, which acts as a buffer in the rumen. As time post feeding progressed, NCTRL had greater ruminal pH when compared with U at h 14 ($P < 0.05$) and NIT at h 20 ($P < 0.04$).

Obvious effects of the different NPN sources on ruminal NH_3-N concentration can be observed in Figure 3-2. All treatments had similar concentration of NH_3-N at 0 h post feeding of molasses, but from h 2 to 6, U and UB had greater concentration of NH_3-N when compared with all other treatments ($P < 0.0001$), and NIT and NITB were greater than NCTRL ($P < 0.02$). Eight hours post feeding, NCTRL still had lesser concentrations of NH_3-N when compared with U and UB ($P < 0.05$), and that difference remained until 10 h post feeding when NCTRL had lesser concentration compared with all other treatments ($P \leq 0.05$). The effect of NPN source on ruminal NH_3-N concentration is one that has been reported by other research groups. Lee et al. (2015b) provided ruminally

cannulated beef heifers with a barley silage-based diet with either 0, 1, 2, or 3% eCAN (DM). All diets were made isonitrogenous with urea. The authors observed a 16% reduction in NH_3-N for 3% eCAN when compared to urea and went on to report that heifers provided eCAN had lesser ruminal NH_3-N concentration at h 3 and 6 postfeeding. Furthermore, El-Zaiat et al. (2014) reported that ruminal concentration of NH_3-N was decreased for Santa Inês lambs consuming eCAN rather than urea in a corn-based diet. In contrast, Veneman et al. (2015), providing dairy cattle urea or calcium-ammonium nitrate, and Nolan et al. (2010), providing Merino weathers oaten chaff with urea or potassium nitrate, both reported no differences between urea and nitrate treatments for concentrations of ruminal NH_3-N. In Chapter 2, reductions in NH_3-N were also observed when CAN was provided compared with urea. The reduction in NH_3-N concentrations in the current experiment may be due to the encapsulation of the nitrate which causes slow release in the rumen; however, Lee et al. (2015b) compared encapsulated urea and encapsulated nitrate and observed similar results. Another possibility is that nitrate may take longer to reduce to NH_3 than urea, which is immediately converted to NH_3 in the rumen, but if this was true, the sustained increase of concentrations of NH_3-N would likely have been observed after the concentrations in the urea treatments reduced to pre-feeding levels; however, this was not detected.

The source of NPN and BSS did not impact concentrations of BUN ($P > 0.05$); however, the addition of NPN to cattle consuming bahiagrass hay and molasses increased concentrations of BUN ($P < 0.0001$). This effect can easily be observed in Figure 3-3. The concentrations of BUN was lesser ($P < 0.05$) for NCTRL when compared with all other treatments at 4, 6, 8, 12, 14, 16, and 20 h post feeding. Just

prior to feeding molasses, NCTRL had lesser concentrations of BUN when compared with NIT and NITB ($P < 0.05$), and U had lesser concentrations of BUN when compared to NITB ($P < 0.05$). Concentrations of BUN was also reduced ($P < 0.05$) in NCTRL when compared with U, UB, and NITB 2 h post feeding of molasses. Also 2 h post feeding, NIT exhibited lesser ($P < 0.05$) concentrations when compared with U and UB, which carried over into 4 h post feeding of molasses. Blood urea N was greater ($P < 0.05$) for U when compared with all other treatments 8 h post feeding. At 10 h post feeding of molasses, NCTRL had lesser ($P < 0.05$) BUN concentration when compared with U, NIT, and UB, and U and UB had greater ($P < 0.05$) concentrations when compared to NITB. Eighteen hours post feeding, NIT had greater ($P < 0.05$) BUN concentration when compared to NCTRL. Finally, 22 h post feeding, NCTRL had less ($P < 0.05$) BUN concentration when compared with U and NITB, and NITB had greater concentration when compared with U, NIT, and UB ($P < 0.05$). No obvious differences between urea and eCAN treatments were observed in the current experiment. This was interesting due to the vast differences in ruminal NH_3-N concentration between urea and eCAN. Contrary to data from the current experiment, Lee et al. (2017b) reported a reduction in BUN for steers provided a silage-based diet with either eCAN rather than urea as a NPN source. The authors followed these same steers to the finishing phase and observed the same patterns where steers receiving urea had greater BUN compared with those consuming eCAN (Lee et al., 2017a).

There were treatment × time interactions observed for acetate ($P = 0.0227$; Figure 3-5) and propionate ($P = 0.0059$; Figure 3-6) molar proportions, and the acetate to propionate ratio (**A:P**; $P = 0.0087$; Figure 3-4). An important aspect of the treatment ×

time interactions observed is the decrease in acetate and increase in propionate molar proportions for approximately 8 h post feeding. This is likely related to an increase in the ruminal concentration of H_2 that is often found shortly after feeding (Janssen, 2010). There were no observed effects of NPN ($P = 0.3585$; Table 3-4) on ruminal concentration of total VFA; however, the molar proportion of acetate was increased at the expense of butyrate when a NPN source was provided ($P < 0.054$; Table 3-5). Similarly, BSS did not affect total VFA concentration ($P = 0.1705$). This is in agreement with data from Chapter 2 Exp. 3 when BSS was included in the substrate DM at 0.33%. Ruiz-Moreno et al. (2015) also reported no difference in total VFA concentration when BSS was included at up to 2% of a high-grain substrate (DM basis) in an in vitro batch culture; however, in the same study, the authors observed a large decrease in VFA concentration when BSS was provided at 1% of the substrate DM in a continuous culture experiment. Furthermore, there was no effect ($P > 0.05$) of BSS on molar proportions of VFA analyzed, which is similar to the results of Exp. 3 from Chapter 2. The addition of eCAN to the bahiagrass hay-based diet reduced ($P = 0.0106$) the concentration of total VFA. This is in agreement with Asanuma et al. (2015) which reported a decrease in total VFA concentration when a nitrate source was provided to goats at 9 g/d. An in vitro experiment utilizing alfalfa hay as the sole substrate had similar results with increasing nitrate concentration decreasing total VFA concentration (Zhou et al., 2012). It should be noted that neither of these studies had isonitrogenous treatments (i.e. no urea control; Zhou et al., 2012; Asanuma et al., 2015). Several others have compared nitrate with urea and reported either no differences (de Raphélis-Soissan et al., 2014; Lee et al., 2015a; Veneman et al., 2015) or increases (Nolan et al.,

2010; El-Zaiat et al., 2014; Zhao et al., 2015) in concentrations of total VFA when a nitrate source was provided rather than urea. It is unclear why total VFA was reduced in the current experiment for cattle receiving eCAN compared with urea. In the current experiment, NH_3-N of all treatments was lesser than what has been recommended in the literature (5.7 m*M*; Satter and Slyter, 1974), more specifically, cattle consuming eCAN had lesser concentrations than those consuming urea; therefore, it is probable that total VFA concentration in the rumen was lesser for eCAN compared with urea in relation to a lack of N for microbial needed for fermentation. The addition of eCAN, in place of urea, also increased ($P = 0.0049$) the molar proportion of valerate when compared to urea.

Effects of BSS and NPN source on digesta and microbial N flow are presented on Table 3-6. Similar to d 13 to 17, DMI and OMI were not affected ($P > 0.05$) by the addition NPN, NPN source, or BSS. Furthermore, flow of DM and OM to the omasum was also not impacted ($P > 0.05$). In humans, NO, an intermediary of nitrate reduction to NH_4, has been reported to cause relaxation of smooth muscle tissue (Butler and Feelisch, 2008). Prior to the commencement of the current experiment, it was speculated that eCAN may have reduce passage rate in ruminants by causing the smooth muscle in the rumen to relax leading to less motility. The data does not support this hypothesis. Although there is very little data regarding bismuth in ruminants, scientists have exhaustedly examined its effects in human and mice models. In mice, bismuth ions have been reported to bind to gastrin compounds inhibiting the effects of the gastrin compound (Pannequin et al., 2004). Gastrin is a peptide hormone that is involved with the secretion of gastric juices and gut motility in monogastrics and also

ruminants (Ozturk et al., 2013). There were numerical reductions in flow to the omasum when BSS was provided; however, no significant differences were observed in the current experiment. Organic matter truly digested in the rumen was not affected by the addition of NPN, NPN source, or BSS ($P > 0.05$).

Microbial N flow to the omasum was not impacted by the addition of NPN, NPN source, or BSS ($P > 0.05$). This is in agreement with Li et al. (2013) who provided Merino lambs with urea or calcium-ammonium nitrate. The authors reported that when sheep were provided a barley-based diet, no differences in microbial N flow were observed. In fact several research groups have reported that microbial N flows are similar for cattle and sheep consuming urea or nitrate as NPN sources (Nolan et al., 2010; Li et al., 2012; Olijhoek et al., 2016). It was unexpected that the addition of NPN did not increase microbial N flow out of the rumen. Researchers have reported that when calcium-ammonium nitrate was provided to sheep consuming oaten chaff (4.1% CP), microbial N flow was increased by more than 160% (Nguyen et al., 2016). It is unclear why no changes in microbial N were observed in the current experiment. Efficiency of microbial N (g/kg of OMTDR) was unchanged ($P > 0.05$) by treatment.

In conclusion, BSS did not have any effects on ruminal fermentation. To the best of the author's knowledge, this is the first data related to in vivo use of BSS in ruminants. Further research should evaluate BSS on ruminal fermentation of cattle consuming high-sulfur, concentrate-based diets. The addition of eCAN had negative effects on ruminal fermentation of cattle consuming bahiagrass hay and molasses. This data indicates that eCAN may not be a viable NPN source for cattle consuming poor-

quality hay-based diets. Future research should inquire about the differences in the effect of nitrate on differing sources and conservation of forages.

Table 3-1. Analyzed[1] chemical composition of the basal diet fed to cattle while in the dry lot and while confined in the UF - NFREC Beef Unit Pavilion

Item	Bahiagrass hay on pasture (average ± SD)	Bahiagrass hay while confined (average ± SD)	Sugar cane molasses (average ± SD)
DM, %	91.6 ± 0.15	94.0 ± 0.52	79.2 ± 0.71
OM, % DM	91.4 ± 0.11	93.9 ± 0.51	84.2 ± 0.35
CP, % DM	9.3 ± 0.25	9.6 ± 0.26	6.8 ± 0.57
NDF, % DM	71.1 ± 0.90	71.9 ± 1.87	-
ADF, % DM	38.5 ± 0.72	39.1 ± 0.27	-
TDN, % DM	54.0 ± 0.0	53.8 ± 0.44	76.5 ± 0.71
Sulfur, % DM	0.24 ± 0.01	0.31 ± 0.01	0.78 ± 0.98
Nitrate, % DM	<0.03	<0.03	0.08 ± 0.06

[1]Analyzed by a commercial laboratory using a wet chemistry package (Dairy One, Ithaca, NY).

Table 3-2. Effect of BSS[1] and eCAN[2] on ruminal gas cap H_2S concentration and rectal temperature

	Treatment[3]						*P*-value[4]				
Item	NCTRL	U	NIT	UB	NITB	SEM[5]	TRT×T	NPN	NS	B	B×N
H_2S[6] d 0, μg/mL	0.44	0.52	0.31	0.39	0.37	0.121	0.788	0.518	0.093	0.599	0.158
H_2S[7] d 1, μg/mL	0.06	0.07	0.03	0.07	0.05	0.021	-	0.999	0.126	0.698	0.658
H_2S[7] d 2, μg/mL	0.13	0.03	0.02	0.05	0.10	0.036	-	0.056	0.678	0.211	0.392
H_2S[7] d 3, μg/mL	0.31	0.09	0.08	0.14	0.11	0.077	-	0.018	0.847	0.632	0.863
H_2S[7] d 14, μg/mL	0.06	0.07	0.07	0.07	0.08	0.012	-	0.532	0.997	0.576	0.757
Temperature[8], °C	38.15	38.40	38.35	38.23	38.15	0.136	0.180	0.057	0.291	0.009	0.826

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: TRT×T = treatment by time interaction; NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided.
[6]H_2S on d 0 was analyzed in the ruminal gas cap at h 0, 3, 6, 9, and 12 post feeding molasses.
[7]H_2S on d 1, 2, 3, and 14 was analyzed in the ruminal gas cap just prior to feeding molasses.
[8]Rectal temperature recorded at 12 time points over 72 h to represent every 2 h post feeding for 24 h.

Table 3-3. Effect of BSS[1] and eCAN[2] on DMI and ruminal fermentation and blood parameters

	Treatment[3]						*P*-value[4]				
Item[5]	NCTRL	U	NIT	UB	NITB	SEM[6]	TRT×T	NPN	NS	B	B×N
Hay DMI, kg/d	4.91	4.94	5.13	5.35	4.59	0.459	-	0.851	0.526	0.884	0.292
Total DMI, kg/d	6.09	6.12	6.31	6.52	5.98	0.475	-	0.767	0.708	0.932	0.429
Ruminal pH	6.66	6.62	6.63	6.66	6.62	0.056	<0.001	0.502	0.690	0.667	0.505
NH_3-N, mM	0.84	3.34	2.08	3.49	2.48	0.565	<0.001	<0.001	0.001	0.375	0.673
BUN, mg/dL	6.41	10.49	9.73	10.33	10.16	0.984	<0.001	<0.001	0.463	0.836	0.640

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: TRT×T = treatment by time interaction; NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]Ruminal fluid and blood samples were collected at 12 time points over 72 h to represent every 2 h post feeding for 24 h.
[6]DMI was recorded from d 13 to 17 of each period when cattle were housed in the UF - NFREC Beef Unit Pavilion.

Table 3-4. Effect of BSS[1] and eCAN[2] on ruminal VFA concentration and A:P[3]

	Treatment[4]						*P*-value[5]				
Item[6]	NCTRL	U	NIT	UB	NITB	SEM[7]	TRT×T	NPN	NS	B	B×N
VFA, m*M*											
Acetate	37.84	41.39	39.10	40.01	37.10	1.590	0.491	0.173	0.018	0.120	0.769
Propionate	9.00	9.81	9.13	9.54	8.67	0.475	0.392	0.373	0.014	0.228	0.753
Butyrate	7.90	7.39	7.04	7.67	7.21	0.500	0.613	0.087	0.190	0.473	0.862
BCVFA[8]	0.25	0.24	0.19	0.25	0.21	0.031	0.324	0.185	0.009	0.267	0.878
Valerate	0.61	0.64	0.82	0.56	0.72	0.098	0.035	0.311	0.025	0.207	0.939
Total VFA, m*M*	55.60	59.49	56.30	58.05	53.94	2.155	0.669	0.359	0.011	0.171	0.734
A:P	4.27	4.27	4.34	4.26	4.36	0.139	0.008	0.715	0.309	0.957	0.981

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]A:P = acetate to propionate ratio.
[4]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[5]Observed significance levels for: TRT×T = treatment by time interaction; NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[6]Ruminal fluid samples collected at 12 time points over 72 h to represent every 2 h post feeding for 24 h.
[7]NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided.
[8]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.

Table 3-5. Effect of BSS[1] and eCAN[2] on ruminal VFA molar proportions

	Treatment[3]						*P*-value[4]				
Item[5]	NCTRL	U	NIT	UB	NITB	SEM[6]	TRT×T	NPN	NS	B	B×N
VFA, mol/100 mol											
Acetate	67.99	69.55	69.34	68.90	68.66	0.868	0.023	0.054	0.670	0.220	0.979
Propionate	16.10	16.43	16.19	16.36	15.97	0.473	0.006	0.710	0.339	0.660	0.818
Butyrate	14.32	12.51	12.61	13.32	13.59	0.823	0.293	0.032	0.735	0.118	0.881
BCVFA[7]	0.44	0.40	0.33	0.43	0.38	0.053	0.217	0.071	0.063	0.155	0.798
Valerate	1.12	1.10	1.50	0.97	1.35	0.181	0.086	0.446	0.005	0.297	0.981

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: TRT×T = treatment by time interaction; NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]Ruminal fluid samples collected at 12 time points over 72 h to represent every 2 h post feeding for 24 h.
[6]NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided.
[7]BCVFA = branched-chain VFA: isobutyrate + isovalerate + 2 methylbutyrate.

Table 3-6. Effect of BSS[1] and eCAN[2] on intake, flow at the omasal canal, and microbial N flow and efficiency

	Treatment[3]						*P*-value[4]			
Item	NCTRL	U	NIT	UB	NITB	SEM[6]	NPN	NS	B	B×N
DM										
Intake, kg/d	5.99	6.19	5.92	5.47	5.17	0.495	0.554	0.553	0.134	0.969
Flow, kg/d	5.16	5.43	4.90	4.77	5.92	0.794	0.911	0.680	0.818	0.276
OM										
Intake, kg/d	5.58	5.77	5.52	5.09	4.80	0.473	0.556	0.551	0.134	0.969
Flow, kg/d	4.16	4.47	3.93	3.86	4.96	0.723	0.849	0.687	0.761	0.242
OMTDR[5], kg/d	1.62	1.56	1.87	1.47	1.00	0.273	0.600	0.754	0.081	0.146
Microbial N flow, g/d	14.33	15.94	14.68	16.41	21.40	2.948	0.365	0.512	0.218	0.275
Microbial efficiency, g of MN[6]/ kg of OMTDR	10.63	8.84	8.77	10.86	13.26	5.549	0.973	0.827	0.549	0.818

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: TRT×T = treatment by time interaction; NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]OMTDR = OM truly digested in the rumen.
[6]MN = microbial N

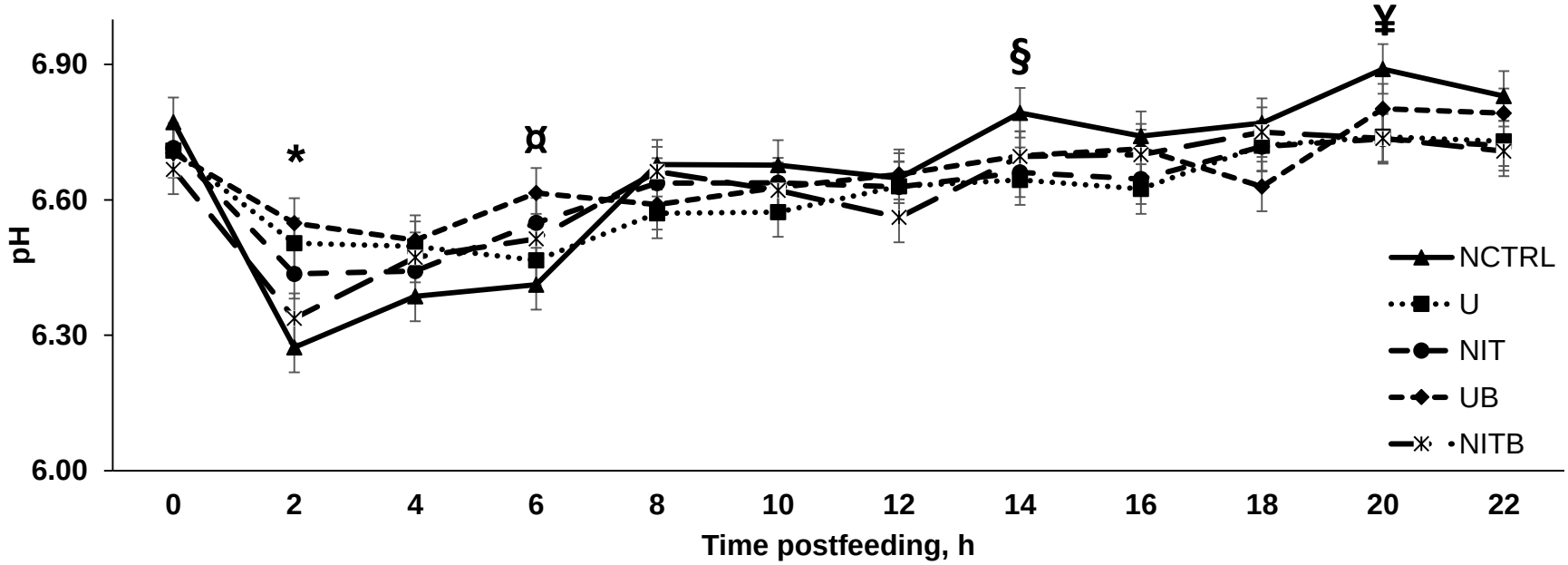

Figure 3-1. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on ruminal pH of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed ($P = 0.0002$). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. * = pH of NCTRL was lesser compared to U, NIT, and UB ($P < 0.03$); U and UB had greater pH than NITB ($P < 0.04$). ¤ = NCRTL and U had lesser pH compared with UB ($P < 0.05$). § = NCTRL had greater pH than U ($P < 0.05$). ¥ = NCTRL had greater pH compared with NIT ($P < 0.04$).

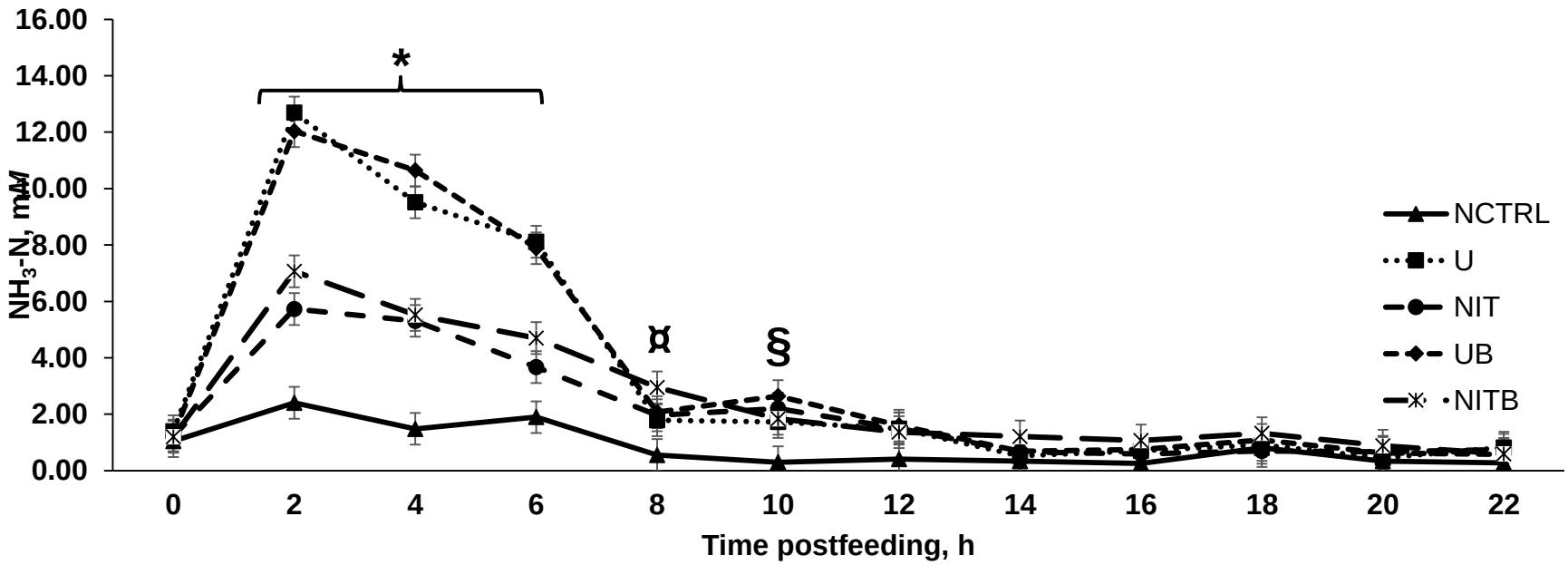

Figure 3-2. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on ruminal NH_3-N concentrations of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed ($P < 0.0001$). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. * = U and UB had greater concentrations of NH_3-N compared to all other treatments ($P < 0.0001$); NIT and NITB had greater concentrations of NH_3-N than NCTRL ($P < 0.02$). ¤ = NCTRL had lesser concentration of NH_3-N compared with UB and NITB ($P < 0.05$). § = NCTRL had lesser concentration of NH_3-N compared with all other treatments ($P \leq 0.05$).

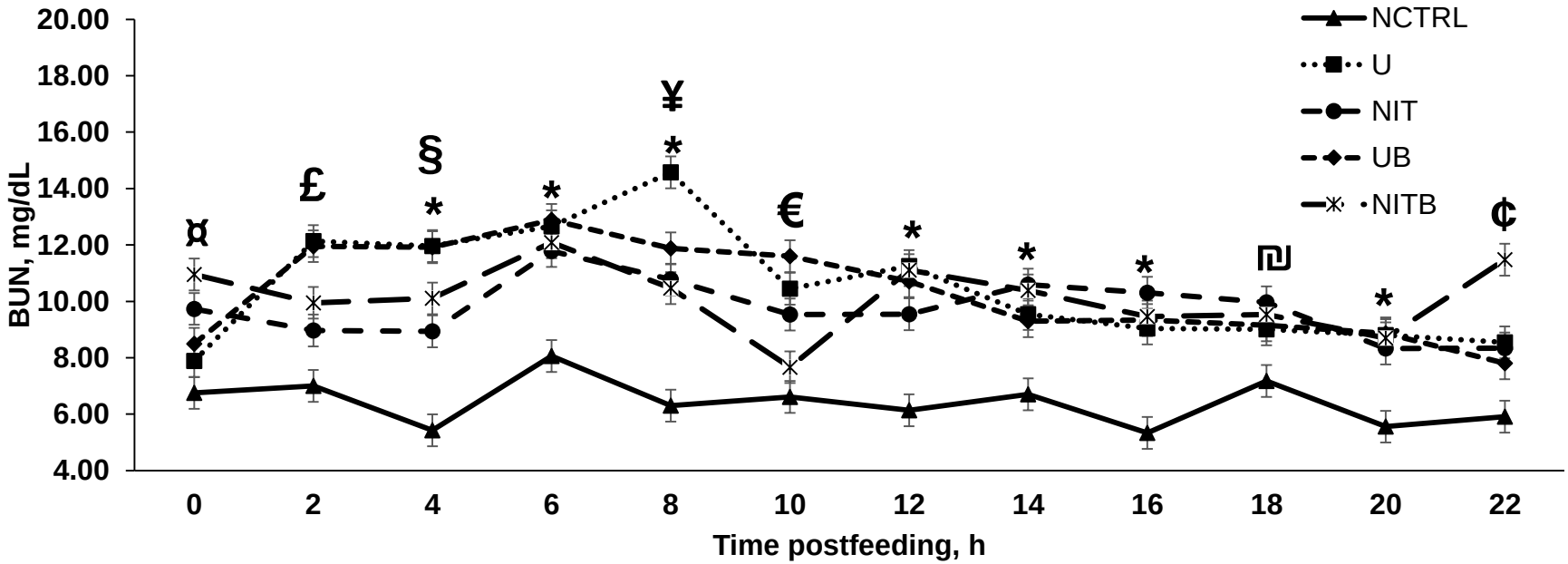

Figure 3-3. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on blood urea nitrogen (BUN) concentrations of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed ($P < 0.0001$). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. ¤ = NCTRL had lesser concentrations of BUN compared with NIT and NITB ($P < 0.05$); and U had lesser concentrations of BUN compared to NITB ($P < 0.05$). £ = NCTRL had lesser concentrations of BUN compared with U, UB, and NITB ($P < 0.05$); and NIT had lesser concentrations compared with U and UB ($P < 0.05$). * = NCTRL had lesser concentrations of BUN compared to all other treatments ($P < 0.05$). § = U and UB had greater concentrations of BUN compared to NIT ($P < 0.05$). ¥ = U had greater concentrations of BUN compared with NIT, UB, and NITB ($P < 0.05$). € = NCTRL had lesser BUN concentration compared with U, NIT, and UB ($P < 0.05$); and U and UB had greater concentrations of BUN compared to NITB ($P < 0.05$). ₪ = NIT had greater BUN concentration compared to NCTRL ($P < 0.05$). ¢ = NCTRL had lesser concentration of BUN compared with U and NITB ($P \leq 0.05$); and NITB had greater concentration of BUN compared with U, NIT, and UB ($P < 0.05$).

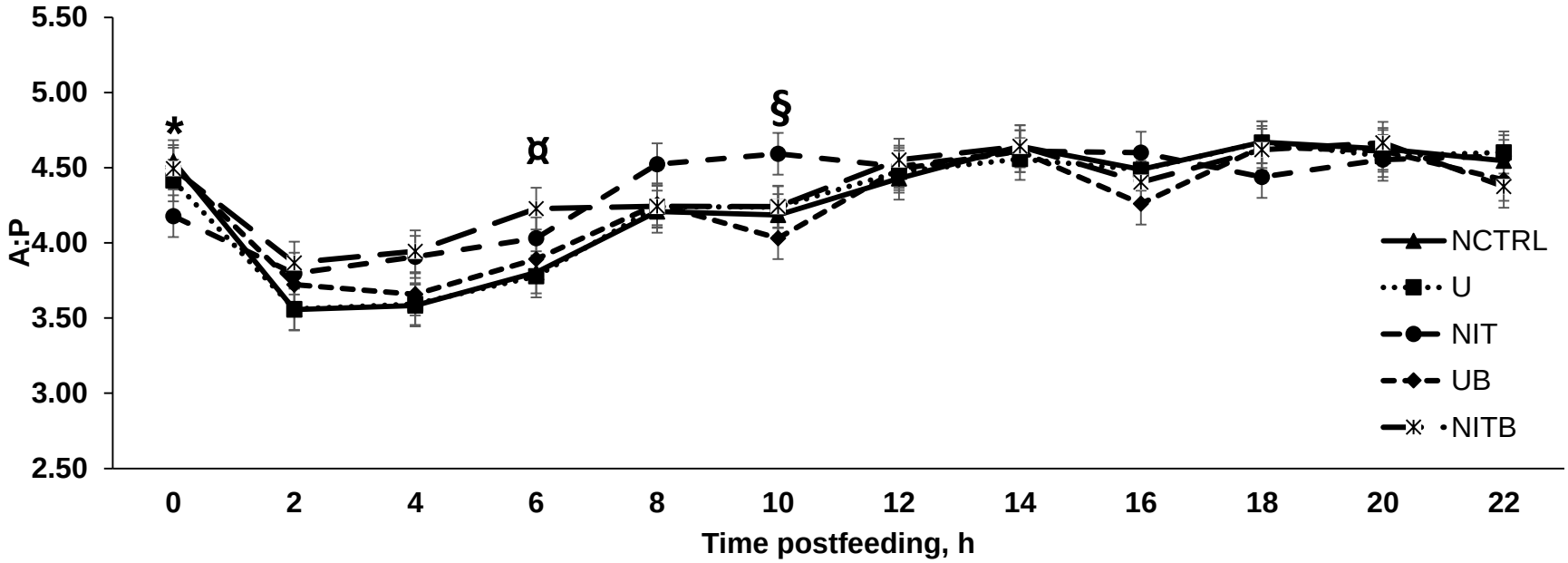

Figure 3-4. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on the ruminal acetate to propionate ratio (A:P) of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed ($P = 0.0087$). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. * = NCTRL had a greater A:P compared with NIT ($P < 0.05$); NIT and NITB had greater concentrations of NH_3-N than NCTRL ($P < 0.02$). ¤ = NCTRL and U had a lesser A:P compared with NITB ($P < 0.03$). § = NCTRL, U, and UB had a lesser A:P compared with NIT ($P \leq 0.05$).

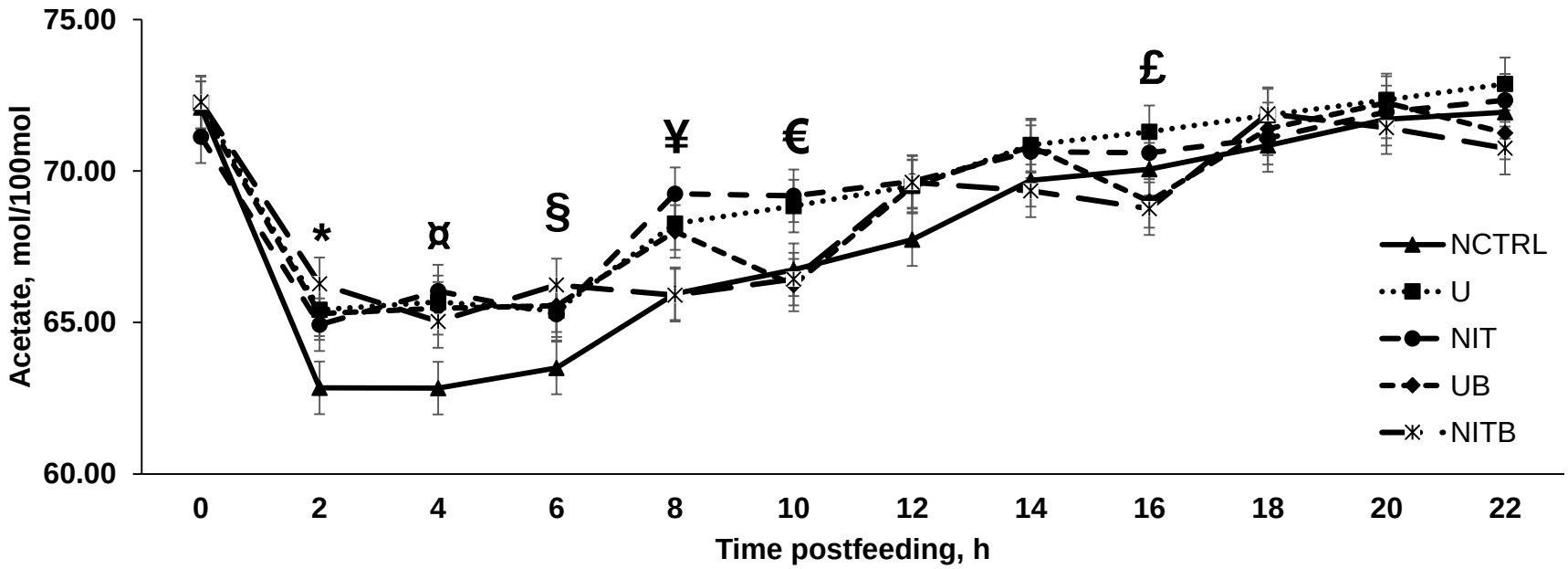

Figure 3-5. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on ruminal acetate molar proportions of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed (P = 0.0227). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. * = NCTRL had lesser concentration than U, UB, and NITB (P < 0.04). ¤ = NCTRL had lesser concentration compared with U, NIT, and NITB (P < 0.03). § = NCTRL had lesser concentration than NITB (P < 0.03). ¥ = NCTRL and NITB had lesser concentrations compared with U and NIT (P < 0.05). € = NCTRL, UB, and NITB had lesser concentrations than NIT (P < 0,03); UB and NITB had lesser concentrations than U and NIT (P < 0.05). £ = U had greater concentration compared with UB and NITB (P < 0.05).

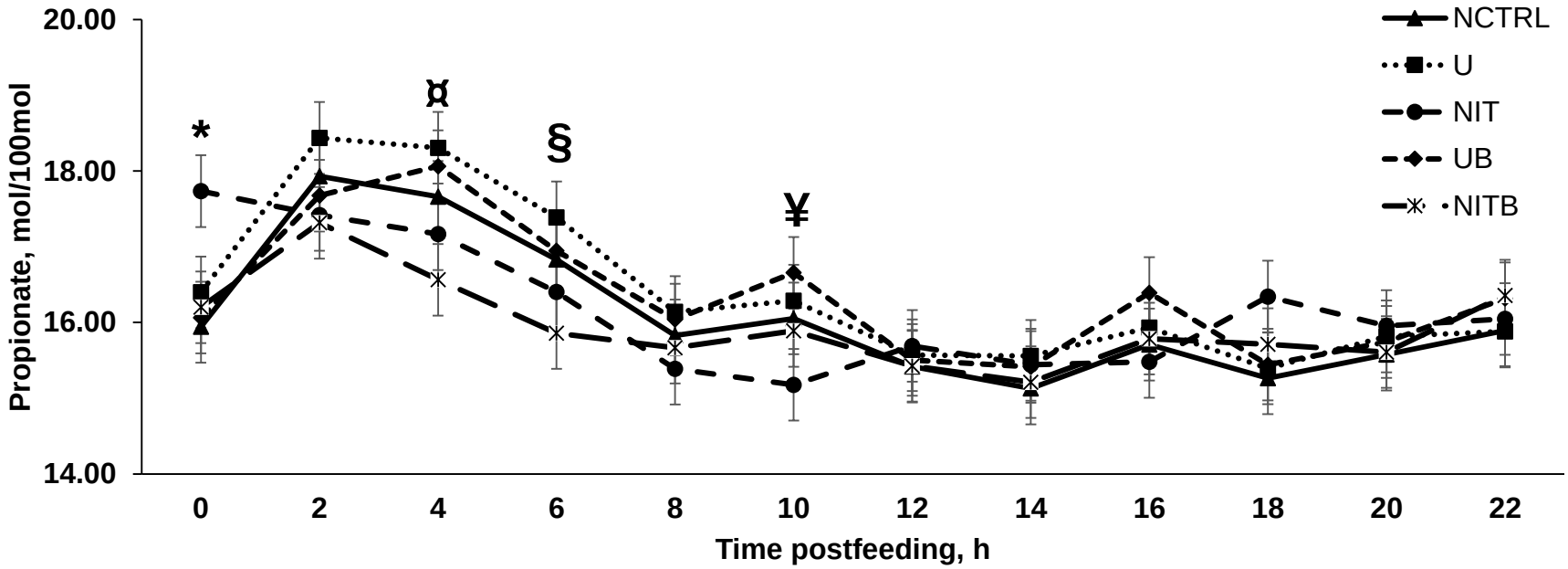

Figure 3-6. Effect of bismuth subsalicylate (BSS) and eCAN (encapsulated calcium-ammonium nitrate; 65.1% N DM basis) on ruminal propionate molar proportions of cannulated Angus-crossbred cattle fed bahiagrass hay ad libitum with 2.7 g/kg BW of molasses. A treatment × time postfeeding interaction was observed ($P = 0.0059$). NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO3-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS. Error bars represent the SEM for treatment × time postfeeding interaction. NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 9, and 8 experimental units, respectively; largest SEM was provided. * = NIT had greater concentration compared to all other treatments ($P < 0.03$). ¤ = NITB had less concentration than U and UB ($P < 0.03$). § = NITB had lesser concentration than U ($P = 0.02$). ¥ = NIT had lesser concentration compared with UB ($P < 0.02$).

CHAPTER 4
EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON ENTERIC METHANE PRODUCTION AND NUTRIENT DIGESTIBILITY OF BEEF STEERS

Introduction

There is a great desire to mitigate enteric CH_4 production of ruminants for environmental concerns and the potential to reduce a portion of GE that would otherwise be lost. Beauchemin et al. (2010) reported that approximately 80% of the total greenhouse gases (**GHG**) produced by the beef industry is related to the cow-calf sector, in which, a majority of cattle are consuming a forage-based diet. Nitrate has been reported to reduce enteric CH_4 production in ruminants consuming several different mixed rations (van Zijderveld et al., 2010; Newbold et al., 2014; Lee et al., 2015b); however, most research disregards the time prior to weaning when 70% of the energy requirements of beef cattle are consumed (Shike, 2013). When considering the ultimate end product of any experiment, the benefit to society, nitrate may be a viable option for cattle producers to provide a needed NPN source while reducing the environmental impact of production.

Bismuth subsalicylate (**BSS**) has been reported to aid in human digestive issues by binding to sulfide in the gastro-intestinal tract, thereby reducing a known gut irritant, H_2S, and by acting as an antimicrobial agent against *Helicobacter pylori*. To the best of the author's knowledge, no research has been published evaluating the effects of BSS on ruminal fermentation, in vivo. Using ruminal fermentation in vitro systems, researchers have observed that BSS can have mitigating effects on H_2S (Ruiz-Moreno et al., 2015); however, no peer-reviewed data has evaluated the effects of BSS on in vitro CH_4 production.

It was hypothesized that the addition of encapsulated calcium-ammonium nitrate (**eCAN**) to a bahiagrass diet supplemented with molasses will reduce enteric methane production; furthermore, BSS may have antimicrobial effects, which may lead to a reduction in CH_4 production. The objective of this experiment was to evaluate the effects of BSS and eCAN, in combination, on apparent total tract digestibility of nutrients, and enteric CH_4 production.

Materials and Methods

All procedures involving animals were approved by the Animal Care and Use Committee of the Institute of Food and Agricultural Sciences at the University of Florida.

Experimental Design, Animals, and Treatments

Twenty-five *Bos taurus* × *Bos indicus* (335 ± 46 kg of BW; average BW ± SD) steers were used in a randomized block design with a 2 × 2 + 1 factorial arrangement of treatments and 2 experimental periods (block) of 20 d each with a 7 d washout period in between where cattle only received bahiagrass (*Paspalum notatum*) hay and sugar cane molasses (chemical composition presented on Table 4-1). On d 0 of each period, BW was recorded to determine the amounts of urea, eCAN, and BSS to provide. From d 0 to 13, steers underwent adaptation to treatment and facilities. Within this 14 d adaptation period, steers receiving NPN were adapted in the following manner: on d 0 and 1, cattle received 20% of their total supplemental N; on d 2 and 3, cattle received 40% of their total supplemental N; on d 4 and 5, cattle received 60% of their total supplemental N; on d 6 and 7, cattle received 80% of their total supplemental N; and beginning on d 8, cattle were receiving 100% of their total supplemental N as urea or eCAN. From d 7 to 13 steers were equipped with training CH_4 collection canisters. Starting on d 13, hay and molasses samples were collected at time of feeding until d 16.

From d 14 to 17 at 0700 and 1500 h, fecal samples were collected. From d 14 to 18, animals were equipped with CH_4 collection canisters. Beginning with the first period, all steers were stratified by weight and breed, and randomly assigned to 1 of 5 treatments: 1) **NCTRL**, no added NPN or BSS; 2) **U**, urea supplemented at 182 mg/kg of BW; 3) **NIT**, nitrate, in the form of eCAN, supplemented at 350 mg/kg of BW; 4) **UB**, urea supplemented at 182 mg/kg of BW and BSS supplemented at 58.4 mg/kg of BW; and 5) **NITB**, nitrate, in the form of eCAN, supplemented at 350 mg/kg of BW and BSS supplemented at 58.4 mg/kg of BW. Treatments U, NIT, UB, and NITB were isonitrogenous. The amount of and adaptation to eCAN and BSS provided was based on the metabolism experiment in Chapter 3 of this dissertation. When these amounts of eCAN and BSS were chosen, it was expected that nitrate would be provided at 2.0% and BSS at 0.33% of the total DMI. After evaluating the DMI during the experimental periods the steers receiving eCAN and BSS consumed 1.54% of the total DMI of nitrate and 0.25% of the total DMI of BSS. After experimental period 1, the 25 steers were re-randomized for experimental period 2, which was identical to experimental period 1 in regards to treatments and procedures.

To ensure that the additional NPN and BSS were consumed in full, urea, eCAN, and BSS were weighed and mixed into the individual steers' molasses daily. All steers had ad libitum access to hay and received 1.07 kg/d of sugar cane molasses. During the experiment, steers were housed 5 per pen in the University of Florida – North Florida Research and Education Center Feed Efficiency Facility in Marianna, FL. Daily DMI of bahiagrass hay was recorded by the GrowSafe system (GrowSafe Systems Ltd.,

Airdrie, Alberta, Canada). Steers were housed in pens of 108 m^2 equipped with 2 GrowSafe feed bunks and a single water trough.

Sampling Procedures

All protocols and procedures used for collecting samples and data from steers were used in an identical manner throughout both experimental periods.

Apparent total tract digestibility of nutrients and retention of S

Apparent total tract digestibility of nutrients was determined using indigestible NDF (**iNDF**) as an internal marker. Feed samples were collected daily from d 13 to 16 and fecal samples were collected at 0700 and 1500 h from d 14 to 17 either by rectal grab or from the ground, inside the pen, immediately after the animal defecated. Hay samples were stored at 10°C, and molasses and fecal samples were stored at -20°C for further analysis. Hay and fecal samples were dried at 55°C for 72 h in a forced-air oven, ground in a Wiley mill (Arthur H. Thomas Co. Philadelphia, PA) to pass a 2-mm screen. Hay was pooled within pen and feces were pooled within steer to determine DM, OM, CP, NDF, ADF, iNDF, and S.

Methane emissions

Emissions of enteric CH_4 production were measured using the sulfur hexafluoride (**SF_6**) tracer technique (Johnson et al., 1994) from d 14 to 18 of each period. Brass permeation tube bodies (length = 4.4 cm, o.d. = 1.43 cm, i.d. = 0.79 cm, inside depth = 3.8 cm, and volume = 1.86 mL), nylon washers, a Teflon membrane, secured with a porous (2-µm porosity) stainless steel frit and a brass nut, were used in each steer. Permeation tubes were filled with approximately 2.3 g of SF_6. Permeation tubes were kept at 39°C and weighed 12 times within 38 d. The average SF_6 release rate across steers was of 2.44 mg/d. Permeation tubes were dosed via balling gun on d 7 of the first

period. Gas collection canisters were constructed of polyvinyl chloride pipe to have a final volume of 2 L. The samples were collected by evacuating the collection canisters to 68.6 cmHg and connecting the canister to a halter, which was equipped with a crimped capillary tube that was positioned to sample, using a loop design, from both nostrils. The volume of the collection canisters and the crimped capillary tubes were designed to allow half of the vacuum to remain after 24 h. Three collection canisters and capillary tubes were used to determine environmental CH_4 and SF_6 concentrations. It has been proposed by Haisan et al. (2014) to consider any animal with at least 2 d of valid CH_4 measurements. For the current experiment, only steers with at least 3 successful d of collection and measurement were considered in the final analysis of CH_4 variables.

Laboratory Analyses

All protocols and procedures used for analyzing samples and data from steers were used in an identical manner throughout both experimental periods.

Concentration of DM, OM, CP, NDF, ADF, and S

All hay and fecal samples were placed in a 55°C forced air oven for 72 h to obtain dry samples. Dry, hot weight was used to calculate DM of the sample. The sample was then ground to pass through a 2-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ). To determine OM, 0.5 g of ground sample (in duplicate) was weighed into ceramic crucibles and placed in a 105°C forced air oven for 24 h to determine sample DM. Dried samples were then placed in a 650°C muffle furnace for 6 h before returning to a 105°C forced air oven. Hot, ashed samples were weighed and used to calculate OM.

Samples of hay and feces were weighed (0.5 g in duplicate) into F57 bags (Ankom Technology Corp., Macedon, NY) and analyzed for NDF, using heat stable α-amylase and sodium sulfite. Subsequent ADF analysis was performed sequentially as described by Van Soest et al. (1991) in an Ankom 200 Fiber Analyzer (Ankom Technology Inc.).

Hay and fecal samples were analyzed for total N and S using a C, H, N, and S analyzer by the Dumas dry combustion method (Vario Micro Cube; Elementar, Hanau, Germany). Crude protein was calculated by multiplying the N concentration of the dry sample by 6.25.

Concentration of iNDF

The concentration of iNDF in hay and feces was determined as described by Gregorini et al. (2008), Cole et al. (2011), and Krizsan and Huhtanen (2013). Briefly, 0.5 g of sample was weighed into Ankom F57 filter bags (Ankom Technology Corp. Macedon, NY) and then incubated into the rumen of a cannulated steer grazing a bahiagrass and bermudagrass (*Cynodon dactylon*) mixed pasture for 288 h to ensure complete digestion of potentially digestible NDF. After incubation, samples were rinsed 2 times with tap water followed by 4 rinses with water filtered through a reverse osmosis system. The rinsed samples were then analyzed for NDF as previously described.

Methane and SF_6 analyses

Methane and SF_6 concentrations in collection canisters were analyzed by gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA). A flame ionization detector and electron capture detector were used for CH_4 and SF_6 analysis, respectively, with a capillary column (Plot Fused Silica 25m by 0.32mm, Coating

Molsieve 5A, Varian CP7536; Varian Inc. Lake Forest, CA). Injector, column, and detector temperatures for CH_4 analysis were 80, 160, and 200°C, respectively. For SF_6, Temperatures were 50, 30, and 300°C for the injector, column, and detector, respectively. The carrier gas for CH_4 and SF_6 was N_2.

Calculations

Emission of CH_4 produced by steers was determined in relation to the SF_6 tracer gas captured in the collection canisters. The following equation was used to quantify CH_4 production:

$$Q_{CH4} = Q_{SF6} \times ([CH_4]_\gamma - [CH_4]_\beta) \div ([SF_6]_\gamma - [SF_6]_\beta)$$

in which Q_{CH4} is considered CH_4 emissions per animal (g/d), Q_{SF6} is considered SF_6 release rate (mg/d), $[CH_4]_\gamma$ is considered the concentration of CH_4 in the animals collection canister, $[CH_4]_\beta$ is considered the concentration of CH_4 in the environmental canisters, $[SF_6]_\gamma$ is considered the concentration of SF_6 in the animals collection canister, and $[SF_6]_\beta$ is considered the concentration of SF_6 in the environmental collection canister.

Apparent total tract digestibility of DM, OM, CP, NDF, and ADF, and retention of S was calculated as follows with OM total tract digestibility as an example:

OM digestibility (%) = 100 – 100 × [(iNDF concentration in the feed ÷ iNDF concentration in the feces) × (OM concentration in the feces ÷ OM concentration in the feed)].

Statistical Analysis

All data were analyzed as a randomized block design with a 2 × 2 + 1 factorial arrangement of treatments using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) using steer as the experimental unit. The model included the fixed effects of

treatment and the random effects of pen and period. To aid in the interpretation of data, the following contrasts were used: the effect of NPN = NCTRL vs. the mean of U, NIT, UB, and NITB; the effect of NPN source = the mean of U and UB vs. the mean of NIT and NITB; the effect of BSS = the mean of U and NIT vs. the mean of UB and NITB; and NPN source × BSS = the mean of U and NITB vs. the mean of NIT and UB. Significance was declared at $P \leq 0.05$.

Results and Discussion

Intake and apparent total tract nutrient digestibility data are presented on Table 4-2. There was no effect ($P > 0.05$) of NPN, NPN source, or BSS on intake of DM, OM, NDF, or ADF during the 4-d digestibility period. This experiment was not designed to test effects on intake; therefore, it cannot be assumed that eCAN and BSS have no impact on DMI. It appears that the effect of nitrate on intake is conditional depending upon diet type and the quality of the diet. Data reported by Lee et al. (2015b) indicated that heifers consuming a diet made of 55% forage (50 and 5% of barley silage and grass hay, respectively) reduced DMI by 6% for when nitrate was used as a source of NPN rather than urea. Lee et al. (2017b) observed that when growing steers were placed on a backgrounding diet with a greater concentration of forage (65% corn silage), DMI was not affected by the inclusion of eCAN at 1.25 or 2.5% of the diet DM. The difference between these two studies may be related to the source of nitrate used: Lee et al. (2015b) provided heifers with an unencapsulated form of calcium-ammonium nitrate, whereas Lee et al. (2017b) used an encapsulated form of the same nitrate. Encapsulation of nitrate has been observed to abate the release of nitrate in vitro, compared to unencapsulated calcium-ammonium nitrate, without affecting NH_3-N or total VFA concentration (Lee et al., 2017c). Likewise, encapsulation of calcium-

ammonium nitrate essentially eliminated nitrite accumulation in vitro where the unencapsulated form allowed accumulation to occur. It is possible that the accumulation of nitrate and nitrite in the rumen may have negative effects on intake of ruminants due to the inhibitory actions on fiber-degrading bacteria (Zhou et al., 2012). Others have reported reductions in DMI of animals consuming 78% concentrate (2.57% calcium-ammonium nitrate; Velazco et al., 2014) and 90% concentrate when provided 2.5% eCAN, but not 1.25% (Lee et al., 2017a). As expected, CP intake during the 4-d digestibility period was increased with the addition of NPN ($P = 0.0021$).

The addition of NPN had no effect ($P > 0.05$) on apparent total tract digestibility of DM, OM, NDF, and ADF. It was hypothesized that nutrient digestibility would have been enhanced with the addition of a NPN source; however, this effect was not observed. The concentration of N in the basal diet was sufficient for microbial growth and development. In the current experiment, the DMI of the NCTRL steers was composed of nearly 9.8% CP, this is a drastic difference from the 4% CP oaten chaff that was provided to Merino ewes by Nguyen et al. (2016). Nguyen et al. (2016) reported that the addition of NPN in the form of calcium-ammonium nitrate increased DM digestibility by 12%. The authors attributed this change in digestibility to an increase in ruminal NH_3-N. It is likely that the NH_3-N concentrations in the rumen of the NCTRL steers provided a sufficient amount of N for the microbial population.

Apparent total tract digestibility of nutrients was not affected by BSS ($P > 0.05$). In a companion in vitro study, presented and discussed in Chapter 2 – Experiments 2 and 3, a linear reduction in in vitro OM digestibility (**IVOMD**) was observed when BSS was included in the substrate DM up to 1.0%. It was speculated in Chapter 2 that this

reduction was one of several indications that BSS was impeding fermentation at a 1.0% inclusion rate; however, in Exp. 3, when BSS was included at 0.00, 0.05, 0.10, or 0.33% of the substrate DM, no effect of BSS on IVOMD was observed. Another research group reported that BSS, included at 1.0% of a high-concentrate substrate, did not negatively affect fermentation (Ruiz-Moreno et al., 2015). In Chapter 2, it was discussed the possibility that the impact of BSS on fermentation was likely diet dependent. Furthermore, it was speculated that when provided with a high-forage diet or substrate, inclusion rates of BSS must be lesser than when provided with a high-concentrate diet or substrate. The data from the current experiment indicates that at approximately 0.25% of DMI of a forage-based diet, BSS does not impact total tract digestibility of nutrients. Further research is needed to evaluate the effects on diets differing in quality and composition.

There was a negative effect of eCAN on DM (P = 0.0152), OM (P = 0.0214), NDF (P = 0.0118), and ADF (P = 0.0125) total tract digestibility. In the literature, there is not a consensus on the impact of nitrate on nutrient digestibility. Researchers have reported increases in DM digestibility when nitrate was included into a 35% forage diet (Li et al., 2013), while others reported no change in nutrient digestibility of diets ranging from 50% (Olijhoek et al., 2016) to 70% forage (Klop et al., 2017; Sun et al., 2017), and, furthermore, linear reductions in DM digestibility have been reported for diets containing 55% forage (Lee et al., 2015b). More research is needed to evaluate the effects of nitrate on nutrient digestibility of cattle consuming a range of diet compositions fed ad libitum and restrictively. The reductions in nutrient digestibility observed in the current experiment may be partially the cause of an accumulation of nitrate/nitrite in the rumen

and/or an increase in the concentration of H_2 in the rumen. It is evident that in the current experiment, the reductions in DM and OM total tract digestibility were largely accounted for by the reduction in digestibility of NDF and ADF. As discussed previously, nitrate and nitrite have toxic effects on certain populations of microbial species in the rumen. Several research groups have observed reductions in the relative abundance of cellulolytic microbial populations in the rumen when nitrate was provided (Marais, 1988; Zhou et al., 2012; Asanuma et al., 2015). These inhibitory effects on fiber-degrading microorganisms would be a probable explanation for the reductions in nutrient digestibility in the rumen. If it was not a direct effect of nitrate/nitrite on microbial populations, it is plausible that the increase in H_2 occasionally observed when nitrate is introduced to a ruminal environment would constrain the activity of certain cellulolytic bacteria (Janssen, 2010; Guyader et al., 2015). It is probable that the reduction in fiber digestion, leading to reduction in DM and OM, was a result of both toxic effects of nitrate/nitrite on ruminal microorganisms and an increase in H_2 concentration in the rumen.

Total tract digestibility of CP was reduced by 8% when eCAN was provided as a NPN source compared with urea (P = 0.0143). It is possible that this is an actual difference and eCAN does reduce the digestibility of CP in the total tract; however, an observation was made when collecting feces from the steers during the digestibility portion of this experiment: eCAN prills were found, intact, in the feces. By observing eCAN prills in the feces of these animals, it was considered that the amount of N analyzed in the feces would be greater, thereby, causing a reduction in the digestibility of CP in the total tract. Other researchers have reported that the percentage of intake N

retention is linearly increased as eCAN is provided up to 3% of DMI (Lee et al., 2015b); however, the heifers used were provided a basal diet that had a greater inclusion of concentrate (45% concentrate) compared with the current experiment (approximately 14% molasses). It is possible that the encapsulation of the nitrate used in the current experiment was over-protected, allowing some nitrate to pass through the total tract unmetabolized. Future research should focus on the effects of differing degrees of protection of nitrate on ruminal fermentation, digestibility, performance, and CH_4 production.

There was no effect ($P > 0.05$) of NPN, NPN source, or BSS on the intake of S during the 4-d digestibility portion of this experiment; however, S retention was reduced ($P = 0.0034$) by 8% with the inclusion of BSS. It has been proposed that BSS in humans reacts in the following manner:

$$2\ Bi^{3+} + 3\ H_2S \leftrightarrow Bi_2S_3 + 6\ H^+.$$

Bismuth sulfide is an insoluble, black salt that, theoretically, is not absorbed through the intestinal wall (Suarez et al., 1998). In the rumen of cattle that are consuming diets which would promote the production of H_2S (high-S, high-concentrate diets; lesser ruminal pH), it may be speculated that BSS acts in a similar manner (Ruiz-Moreno et al., 2015); however, it is not evident what reaction is occurring when H_2S is not present, or is present in extremely reduced concentrations, such as that observed in the metabolism experiment presented and discussed in Chapter 3. Whatever the mode of action, the data indicates that BSS is shifting a portion of the intake S that would be apparently retained to pass though the gastro-intestinal tract and be expunged in the feces of the animal.

Methane production parameters are presented on Table 4-3. It has been widely accepted that nitrate reduces enteric methane emissions of ruminants (Hulshof et al., 2012; Newbold et al., 2014; Lee et al., 2015b). This reduction in CH_4 production is thought to be related to 2 factors: nitrate acting as a H_2 sink (Leng, 2008), and the inhibitory effects of nitrate, more specifically nitrite, on methanogens (Duin et al., 2016). In the current experiment, a reduction (P = 0.0393) in CH_4 (g/d) was observed when eCAN was provided as a NPN source rather than urea. When evaluating grams of CH_4 produced per kilogram of OM intake and OM digested, there was no effect of NPN source (P > 0.05). Although there was not a statistical difference in OM among the steers, which consumed eCAN rather than urea, there was a numerical reduction, and since this CH_4 variable (g/kg OM intake) is calculated, the similarity among treatments is reasonable. Furthermore, when the effect of OM digestibility was considered, eCAN was unsuccessful at reducing emission intensity. When metabolic BW was taken into account, eCAN reduced (P = 0.0513) CH_4 production (g/kg of $BW^{0.75}$) by 11% compared with urea. There was no effect of NPN or BSS on any of the CH_4 variables evaluated in this experiment.

In conclusion, the data indicate that the addition of BSS does not negatively affect apparent total tract digestibility of nutrients, nor does BSS appear to mitigate enteric CH_4 emissions. Further research is needed to evaluate the effects of BSS on differing dietary compositions in regards to nutrient digestibility. It may be plausible in the future to provide BSS to finishing cattle consuming byproducts, such as distiller's grains, to mitigate the negative effects associated with high-S diets. The inclusion of eCAN in the diets of ruminants provided forage-based diets may reduce daily emissions

of CH_4 (g/d); however, the effect of nitrate on digestibility should be considered. Future research should focus on the effects of nitrate on grazing cattle and cattle consuming different forage types.

Table 4-1.Analyzed chemical composition of bahiagrass hay and sugar cane molasses

Item	Bahiagrass hay (average ± SD)	Sugar cane molasses[1] (average ± SD)
DM, %	88.8 ± 1.69	79.2 ± 0.71
OM, % DM	94.9 ± 0.18	84.2 ± 0.35
CP, % DM	10.2 ± 0.72	6.8 ± 0.57
NDF, % DM	71.8 ± 2.87	-
ADF, % DM	35.2 ± 3.22	-
Sulfur, % DM	0.29 ± 0.02	0.78 ± 0.98
Nitrate, % DM	-	0.08 ± 0.06

[1]Analyzed by a commercial laboratory using a wet chemistry package (Dairy One, Ithaca, NY).

Table 4-2. Effect of BSS[1] and eCAN[2] on nutrient intake and apparent total tract digestibility, and sulfur intake and retention

	Treatment[3]						*P*-value[4]			
Item	NCTRL	U	NIT	UB	NITB	SEM[5]	NPN	NS	B	B×N
4-d intake, kg/d										
DM	8.31	8.17	7.79	8.43	7.92	0.442	0.636	0.321	0.666	0.887
OM	7.76	7.63	7.26	7.87	7.39	0.418	0.632	0.320	0.666	0.888
CP	0.81	0.98	0.94	1.00	0.96	0.043	0.002	0.353	0.714	0.953
NDF	5.11	5.00	4.71	5.18	4.80	0.323	0.609	0.306	0.674	0.907
ADF	2.52	2.46	2.30	2.54	2.35	0.162	0.576	0.288	0.690	0.931
S	0.039	0.038	0.037	0.039	0.037	0.001	0.627	0.290	0.727	0.963
Digestibility, %										
DM	52.58	52.99	51.65	52.66	50.66	0.658	0.424	0.015	0.323	0.620
OM	54.09	54.87	53.59	54.28	52.69	0.601	0.727	0.021	0.222	0.795
CP	34.66	46.82	43.67	46.14	41.89	1.444	<0.001	0.014	0.401	0.707
NDF	52.09	52.22	50.81	52.40	50.98	0.536	0.419	0.012	0.744	0.990
ADF	49.36	50.16	47.25	50.25	49.30	0.737	0.881	0.013	0.155	0.193
S retention[6], %	61.94	62.41	61.31	57.49	56.01	1.642	0.158	0.437	0.003	0.908

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]NCTRL, U, NIT, UB, and NITB had 10, 10, 10, 10, and 10 experimental units, respectively; largest SEM was provided.
[6]S absorption was calculated as follows: 100 – (100 × (([diet iNDF] ÷ [fecal iNDF]) × ([fecal S] ÷ [diet S]))).

Table 4-3. Effect of BSS[1] and eCAN[2] on enteric CH_4 production of beef steers

	Treatment[3]						*P*-value[4]			
Item[5]	NCTRL	U	NIT	UB	NITB	SEM[6]	NPN	NS	B	B×N
CH_4 emissions, g/d	121.16	117.27	115.57	134.09	100.04	10.986	0.673	0.039	0.951	0.121
CH_4 emissions, g/kg OMI[7]	15.35	15.42	16.44	15.59	13.67	1.197	0.954	0.689	0.264	0.209
CH_4 emissions, g/kg OMD[8]	28.72	28.63	30.33	29.36	25.70	2.246	0.921	0.649	0.372	0.222
CH_4 emissions, g/kg MBW[9]	1.51	1.39	1.44	1.55	1.18	0.126	0.341	0.051	0.689	0.063

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]CH_4 was determined from the average of at least three out of five 24-h periods of breath sample collection.
[6]NCTRL, U, NIT, UB, and NITB had 10, 8, 7, 8, and 7 experimental units, respectively; largest SEM was provided.
[7]OMI = OM intake.
[8]OMD = OM digested.
[9]MBW = $BW^{0.75}$.

CHAPTER 5
EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON LIVER MINERAL CONCENTRATION AND PERFORMANCE OF GROWING BEEF HEIFERS

Introduction

There is a growing desire to reduce the environmental impacts of beef production. One methodology that has been rigorously evaluated and reported is the addition of nitrate to the diets of cattle in place of traditional urea (van Zijderveld et al., 2011; Newbold et al., 2014; Hegarty et al., 2016). Most data indicate that nitrate can reduce enteric CH_4 production by 10 to 30% (Lee and Beauchemin, 2014; Guyader et al., 2015). Enteric CH_4 production can account for 2 to 12% of GE losses, depending upon diet type, and it has been theorized that nitrate may increase ME supply by reducing the amount of C lost as CH_4; however, most research has focused on the performance of cattle consuming moderate- (Lee et al., 2017b) to high-concentrate (Newbold et al., 2014) diets.

Little is known about the effects of bismuth subsalicylate (**BSS**) on ruminant animals. For decades, BSS has been heralded as a mediator of H_2S in humans, lessening pain in the gastro-intestinal tract (Suarez et al., 1998; Levitt et al., 2002; Mitsui et al., 2003). In vitro ruminal fermentation has been used to evaluate the possible influence of BSS on in vivo parameters, but in vivo data is needed to truly evaluate to potential impacts on production and performance of cattle (Ruiz-Moreno et al., 2015). Bismuth compounds may have a place in beef production by mitigating the negative effects of S on trace mineral absorption. By binding to S (Suarez et al., 1998), BSS may reduce thiol- compounds which inhibit trace mineral absorption.

The current experiment was designed to test the hypothesis that encapsulated calcium-ammonium nitrate (**eCAN**) would not impact performance of growing heifers consuming a bahiagrass hay and molasses diet. The second hypothesis was that BSS would not alter performance of growing heifers; however, liver trace mineral content should be increased. The objective of this experiment was to evaluate the performance and liver mineral concentration of heifers provided eCAN and/or BSS.

Materials and Methods

All procedures involving animals were approved by the Animal Care and Use Committee of the Institute of Food and Agricultural Sciences at the University of Florida.

Experimental Design, Animals, and Treatments

Seventy-five growing *Bos taurus* and *Bos indicus* heifers (279 ± 57 kg of initial BW; average ± SD) were used in an randomized incomplete block design with a 2 × 2 + 1 factorial arrangement of treatments at the University of Florida – North Florida Research and Education Center Beef Unit in Marianna, FL. The experiment consisted of a 28 d adaptation period followed by a 56 d data collection period in which heifers were weighed every 14 d from d 0 to d 56. On d -28 and -27, all heifers were weighed and the average weight of each heifer on those 2 d was considered initial BW. Similarly, the average BW of each heifer on d 55 and 56 was considered final BW. On d -27, heifers were stratified and blocked by weight and allotted to 25 dormant bahiagrass (*Paspalum notatum*) pastures. Pastures (1.34 ha each) were located in 3 different areas of the Beef Unit and were within 0.52 km of each other. The three locations were termed North Circle (n = 13; pastures per location), South Circle (n = 6), and R-Pens (n = 6). Pastures were stratified by location and randomly assigned to 1 of 5 treatments: 1) **NCTRL**, no added NPN or BSS; 2) **U**, urea supplemented at 182 mg/kg of BW; 3) **NIT**, nitrate, in the

form of eCAN, supplemented at 350 mg/kg of BW; 4) **UB**, urea supplemented at 182 mg/kg of BW and BSS supplemented at 58.4 mg/kg of BW; and 5) **NITB**, nitrate, in the form of eCAN, supplemented at 350 mg/kg of BW and BSS supplemented at 58.4 mg/kg of BW. Treatments U, NIT, UB, and NITB were isonitrogenous.

To reduce any negative effects of nitrate on the health of the heifers, on d -27 cattle began an adaptation to eCAN and urea. From d -27 to d -14, cattle received 20% of their total supplemental N; on d -13 and -12, cattle received 40% of their total supplemental N; on d -11 and -10, cattle received 60% of their total supplemental N; on d -9 and -8, cattle received 80% of their total supplemental N; and beginning on d -7, cattle were receiving 100% of their total supplemental N as urea or eCAN. One pasture (NITB) was removed from the experiment because the heifers refused to consume the molasses and treatment.

Heifers had ad libitum access to bahiagrass hay and received 1.07 kg/d (DM basis) of sugar cane molasses (chemical composition is presented on Table 5-1). This experiment began on February 15, 2017 and prior to initiation, pastures were mob grazed to remove any residual forage; therefore, bahiagrass hay was the only forage available to the heifers. Molasses was weighed and provided daily, and was used as the carrier of treatments.

Sample and Data Collection

Samples of hay and molasses were collected every 14 d and stored at 10°C (hay) or -20°C for further analysis. All feed samples were shipped to a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY) for chemical analysis, which are presented in Table 5 - 1.

Heifers BW was recorded every 14 d starting on d 0. To calculate ADG, difference in BW was divided by the number of d between BW recordings.

Liver samples were taken on d -28 and -27, and again on d 55 and 56. A random subset of heifers (2 heifers/pen) was selected to collect liver tissue. One heifer per pen was randomly selected to have liver sample taken on d -28 and 55, and the second heifer donating liver tissue was collected on d -27 and 56. There were no statistical differences ($P > 0.05$) between cohorts; therefore, for the rest of the manuscript, liver tissue will be described as initial and final. The techniques described in Arthington and Corah (1995) were used to collect liver tissue. Briefly, A Tru-Cut biopsy needle (CareFusion, 14 gauge by 15 cm; Becton, Dickinson, and Comp., Vernon Hills, IL) was inserted between the 11th and 12th intercostal space on each animal until Glisson's capsule was penetrated. Three core tissue samples (25 mg each) were collected from each animal at each collection. All tissue samples were stored in Zn free microcentrifuge tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) at -20°C until further analysis. Liver tissue mineral concentration was determined using ICP-MS (Animal Health Diagnostic Laboratory, Lansing, MI).

On d -28, -27, 55, and 56, carcass measurements were taken using ultrasonography (3.5-mHz linear array transducer, Aloka 500V; Corimetrics Medical Systems Inc., Wallingford, CT). Two-thirds of the heifers were measured on d -28 and 55 (all animals not donating liver tissue plus the first cohort of liver tissue donors) and one-third was measured on d -27 and 56 (the second cohort of liver tissue donors). No differences were found between d -28 and -27, nor d 55 and 56; therefore, for the remainder of the manuscript, ultrasonography measurements will be described as initial

and final. Ultrasonography measurements were made between the 12th and 13th intercostal space. Images were used to assess LM area and back fat thickness.

Statistical Analysis

Data were analyzed as a randomized incomplete block design with a 2 × 2 + 1 factorial arrangement of treatments using pasture as the experimental unit. The model included the fixed effect of treatment and the random effects of block and location (North Circle, South Circle, and R-Pens). Pens blocked (n = 5) by BW. Initial liver mineral concentration and ultrasound measurements were used as covariates for final liver mineral concentration and ultrasound measurements, respectively. Denominator degrees of freedom were adjusted using the Kenward-Rogers adjustment. The following contrasts were used to aid in the interpretation of data: the effect of NPN = NCTRL vs. the mean of U, NIT, UB, and NITB; the effect of NPN source = the mean of U and UB vs. the mean of NIT and NITB; the effect of BSS = the mean of U and NIT vs. the mean of UB and NITB; and NPN source × BSS = the mean of U and NITB vs. the mean of NIT and UB. Significance was declared at $P \leq 0.05$.

Results and Discussion

This experiment was the final of a series of experiments to examine the effects of providing eCAN as a NPN source along with BSS. Data from the previous experiments indicated that rumen fermentation was altered by the addition of eCAN, inplace of urea, but not BSS. Furthermore, digestibility and enteric CH_4 production (g/kg of $BW^{0.75}$) was reduced with the addition of eCAN. In the current experiment, treatments identical to those used in Chapters 3 and 4 were provided to the heifers to consider the consequences producers may face if these compounds are fed commercially.

Growth performance data is presented on Table 5-2. At the beginning of the current experiment, all treatments had similar BW ($P > 0.05$). By the end of the experiment, BW was not affected by NPN ($P = 0.4701$), NPN source ($P = 0.3836$), or BSS ($P = 0.5999$); however, there was an interaction ($P = 0.0484$) between BSS inclusion and source of NPN. There was no effect of NPN, NPN source, BSS or an interaction for ADG measured within any time points ($P > 0.05$). As discussed in Chapter 2, it is possible that the CP content of the forage and molasses was sufficient to support the growth and development of a healthy and productive ruminal microbiome. The most recent NRC model (NASEM, 2016) recommends a CP content of the diet to range from 10.2 to 17.2% of the diet DM for growing animals weighing 250 to 350 kg. This range of CP concentration depends greatly on the energy content of the ration. In the current experiment, assuming cattle were consuming 85% bahiagrass hay and 15% molasses (see Chapter 4), the CP content of the diet would have been approximately 9.8% of the DMI with nearly 57.5% TDN. For growing animals, the NRC gives examples and recommendations using 4 different diets ranging from 65 to 80% TDN. With less TDN in the diet of the current experiment compared with the NRC examples, it may be that the animals could not synchronize the use of all of the available CP due to a lack in fermentable energy (Dewhurst et al., 2000; NASEM, 2016). In a review manuscript by Dewhurst et al. (2000), the theory of rumen synchrony was discussed in depth. The authors deliberated over several data sets evaluating diets that differed in rumen available energy and N sources. The authors concluded that a balance between fermentable energy and N sources should be sought after to optimize microbial protein synthesis. It could be speculated that in the current experiment, the energy available for

microbial protein synthesis was not sufficient to utilize the NPN sources provided; thereby, not improving performances of animals provided NPN.

When considering the proposed mode of action of the reduction of CH_4 production with the addition of nitrate, it may be speculated that performance of the ruminant being provided nitrate would increase. When nitrate is reduced in the rumen to NH_3, H_2 that would be used to reduce CO_2 to CH_4 would theoretically no longer be available; therefore, possibly a shift in the VFA profile to produce more propionate would occur, leading to increases in performance. Data from the current experiment does not support this theory nor does a majority of the literature on nitrate report such findings (Newbold et al., 2014; Lee et al., 2017a; Lee et al., 2017b). It has been hypothesized that the production of propionate is not increased with the addition of nitrate because of the thermodynamics of VFA production (Ungerfeld and Kohn, 2006; Janssen, 2010; Ungerfeld, 2015). To speculate on this subject, one must assume that nitrate only acts on enteric CH_4 production by acting as a H_2 sink only and not inhibiting methanogens. When nitrate is reduced in the rumen, conceptually, one can visualize the pool of H_2 to be lessened. In a review by Janssen (2010), evidence was presented that indicated a shift in VFA production to H_2 producing pathways (i.e. 2 acetate + 4 H_2); therefore, increasing the amount of C used for acetate production at the expense of butyrate and/or propionate. It is plausible that this occurred in the current experiment and C that would have been lost as CH_4 was used to produce acetate, a VFA less energetically favorable than propionate. Future research should focus on the long-term effects of nitrate on ruminal fermentation to evaluate if the shift in VFA, as seen in Chapter 3, persists over a longer forage feeding period.

To the best of the author's knowledge, this is the first data set describing the performance of ruminants provided BSS. A plethora of data has been reported indicating the negative effects of S on beef cattle performance (Gould, 1998; Felix et al., 2012; Drewnoski et al., 2014). It was hypothesized that BSS may be able to thwart these negative effects by binding to ruminal S and transporting it, as Bi_2S_3, out of the gastro-intestinal tract via feces. In the current experiment, the diet consisted of approximately 0.51% S (DM basis), which is marginally greater than what is considered safe for grazing beef cattle (NASEM, 2016). Early research has indicated that growing cattle consuming forage-based diets and high-S water (approximately 0.55% S in DMI) experience reductions in DMI and BW gain (Weeth and Hunter, 1971; Weeth and Capps, 1972). In the current experiment, heifers not receiving BSS did not perform less favorably compared with those which were provided BSS, indicating that heifers were not affected by the S concentration of the diet and/or BSS did not improve performance by sequestering S in the rumen. Data from a ruminal metabolism experiment, presented and discussed in Chapter 3, indicated that BSS had minimal effects on the concentration of H_2S in the gas cap of ruminally cannulated cattle consuming bahiagrass hay and molasses (0.40% S DM basis). Previous work has indicated that the activity of BSS is impacted by pH, with more acidic environments being more appropriate (Sox and Olson, 1989). It is possible that the rumen environment was not as conducive for BSS activity in the current experiment compared to a ruminant fed a high-grain diet. Researchers published data from an in vitro batch culture experiment using a 90% concentrate substrate with 1% BSS (DM basis), in which fermentation was not negatively impacted by BSS, while H_2S was reduced by 34% indicating that BSS was

binding to S (Ruiz-Moreno et al., 2015); however, in a separate in vitro continuous culture experiment, the same inclusion rate of BSS reduced H_2S by nearly 99%. When evaluating the pH of each in vitro experiment, the batch culture had a final pH of approximately 6.3, whereas the mean pH of the continuous culture was approximately 5.4 (Ruiz-Moreno et al., 2015). An in vitro experiment, presented and discussed in Chapter 2, was used to evaluate BSS at up to 1.0% of the DM of a bahiagrass hay and molasses substrate. At 1.0%, H_2S was reduced by 100%; however, it was obvious from the results of other variables, that fermentation had been negatively affected. When included in the substrate at 0.33%, BSS reduced H_2S by 61% without negatively affecting in vitro fermentation. Currently, there is not enough data to conclude exactly what is occurring in the rumen when BSS is provided, but the data from the current experiment does not support the hypothesis that BSS will improve the performance of cattle consuming high-S, forage-based diets.

Suarez et al. (1998) theorized that BSS lessened gastro-intestinal pain in humans by binding to H_2S, a major gut irritant in humans, in the following manner:

$$2\ Bi^{3+} + 3\ H_2S \leftrightarrow Bi_2S_3 + 6\ H^+.$$

If this hypothesis is correct, then it is possible that bismuth only binds to sulfide when it is in the undissociated form. This may explain the interaction observed between NPN source and BSS. In Chapter 2 – Exp. 2, the inclusion of a nitrate source reduced H_2S in a linear manner. Although no effects of NPN source on ruminal H_2S concentration were observed in the metabolism experiment (Chapter 3), if eCAN reduced the amount of H_2S produced in the current experiment, by acting as a H_2 sink, the pool of H_2 would likely be comparably less. Speculatively, if BSS could not bind to H_2S, it may be more

likely to act as an antimicrobial (Sox and Olson, 1989) and inhibit growth of beneficial bacterial populations in the rumen.

Carcass ultrasound results are presented on Table 5-3. There was no effect of NPN, NPN source, or BSS on LM area ($P > 0.05$). As previously discussed, there was an expectation for NPN to improve performance, thereby, possibly increasing LM area; however, this was not observed in the current experiment. The lack of change in LM area when eCAN was provided, in place of urea, is in agreement with much of the literature (Hegarty et al., 2016; Lee et al., 2017a). In general, the benefits of nitrate on performance are observed in increases in G:F (Newbold et al., 2014; Lee et al., 2017a). As BSS was never reported to have been provided to ruminants, the data from this experiment provides a good base for other researchers to build on. Surprisingly, a BSS × NPN source interaction ($P < 0.001$) was observed for 12th–rib fat thickness on d 56 and change in fat thickness from d -28 to d 56. The data from this experiment indicates that BSS may increase fat deposition when urea is provided as a NPN source; however, potentially negative effects on fat thickness may occur when BSS is provided with eCAN. Although not significant, butyrate molar proportions were numerically increased, in the metabolism experiment presented and discussed in Chapter 3, when BSS was provided. If the heifers in the current experiment encountered shifts in ruminal fermentation to produce more butyrate, it is possible that more butyrate was utilized in energy production through the tricarboxylic acid cycle (Van Soest, 1994). Furthermore, by providing NPN, fat thickness at the 12th–rib was increased ($P = 0.042$).

The concentration of trace minerals in the liver from cattle in the current experiment can be found on Table 5-4. Concentration of minerals in the liver was not

affected by NPN source ($P > 0.05$). To the best of the author's knowledge, this is the first data representing the effects of nitrate supplementation on liver mineral concentration. There were no interactions between BSS and source of NPN ($P > 0.05$) affecting liver mineral concentration. The addition of NPN increased liver concentrations of Fe ($P = 0.0132$) and Mn ($P = 0.0056$) by 37 and 15%, respectively. The reason for this change in Fe and Mn is unknown.

It was hypothesized that BSS may increase the liver concentration of trace minerals when BSS was provided with a high-S diet. The effects of diets with large concentrations of S on trace mineral absorption has been reported in both grazing cattle (Arthington et al., 2002) and grain-fed cattle (Pogge and Hansen, 2013; Pogge et al., 2014). In the rumen, S, along with Mo, can act as an antagonist to trace mineral (i.e. Cu) absorption. The S binds with Mo to form thiomolybdates that bind to minerals, leaving them inabsorbable (Drewnoski et al., 2014). It was speculated that BSS may reduce the amount of available S in the rumen by forming Bi_2S_3. Without having available S to bind to trace minerals, the minerals, theoretically, would have a greater chance of absorption. The data from this experiment does not support this hypothesis. Liver mineral concentrations of Cu were reduced ($P = 0.0018$) by 70% when BSS was provided to heifers consuming bahiagrass hay and molasses. Unfortunately, very little data is available evaluating the effects of bismuth on the mineral status of humans and rats. One study injected $BiCl_3$ subcutaneously into rats to evaluate the effects of bismuth on Cu concentration of differing rat organs (Szymanska and Zelazowski, 1979). The researchers found an increase in total body Cu content with no change in liver concentrations of Cu; however, the concentration of Cu observed in the kidneys was

increased with the injection of bismuth. It may be speculated that if kidney concentrations of Cu are increased, that an increase in urine Cu may be observed; however, the authors did not evaluate such parameters (Szymanska and Zelazowski, 1979). Assuming that bismuth causes a shift in Cu transportation to the kidneys, it may be possible that the heifers receiving BSS in the current experiment experienced this change in Cu accumulation. Another possibility is that formations of Bi-Cu-S compounds bound Cu, and excreted the Cu via feces. Researchers have discussed the synthesis of Cu_3BiS_3 nanocrystals in the laboratory (Yan et al., 2013); however, there is no evidence that this can occur naturally in the rumen.

The inclusion of BSS in the diets of heifers was also associated with a 30% increase (P = 0.0157) in liver concentration of Fe. There has been some evidence that bismuth may act as an antimicrobial by inhibiting bacterial uptake of Fe (Domenico et al., 1996). More recently, other research groups have contested this theory claiming that the effects of bismuth on bacteria (i.e. *Helicobacter pylori*) is similar to intracellular iron deprivation and causative (Bland et al., 2004). Further research needs to focus on the mode of action of bismuth on *H*. *pylori*. If there is a link between bismuth and the uptake of Fe by bacteria, it is possible that more Fe is available for absorption in the duodenum; however, this is unlikely. It is more probable that the increase in liver Fe concentration is related to the depletion of Cu in the liver (Suttle, 2010). Copper is vital for the activity of ferroxidase, an enzyme which mobilizes Cu out of the liver; therefore, if Cu is limiting in the liver, Fe is likely to accumulate in the hepatic tissue (Mills et al., 1976; Hansen et al., 2010; Suttle, 2010).

In conclusion, the inclusion of eCAN did not improve nor worsen performance of growing heifers unless it was provided in combination with BSS. Alone, BSS did not hinder growth of heifers consuming bahiagrass hay and molasses. The diet in the current experiment contained approximately 0.5% S (DM) which may not have been great enough to truly observe the potential benefits of BSS on performance. Regardless of performance, heifers consuming BSS exhibited reductions in liver Cu and liver Fe accumulation, which may have detrimental effects in the long term. More research is required to determine the effects of BSS on cattle consuming diets of differing composition and S content.

Table 5-1. Analyzed[1] chemical composition of bahiagrass hay and sugar cane molasses

Item	Bahiagrass hay	Sugar cane molasses
DM, %	90.0	78.7
OM, % DM	93.4	84.4
CP, % DM	10.3	7.2
NDF, % DM	73.0	-
ADF, % DM	45.7	-
TDN, % DM	54.0	77
S, % DM	0.34	1.47
Nitrate, % DM	0.07	0.12

[1]Analyzed by a commercial laboratory using a wet chemistry package (Dairy One, Ithaca, NY).

Table 5-2. Effect of BSS[1] and eCAN[2] on growth performance of beef heifers

	Treatment[3]					*P*-value[4]			
Item	NCTRL	U	NIT	UB	NITB	SEM[5]	NPN	NS	B	B×N
Initial BW[6], kg	274	275	276	279	277	3.1	0.349	0.930	0.435	0.615
Final BW[7], kg	335	335	342	352	332	6.7	0.470	0.384	0.600	0.048
ADG, kg										
d -28 to 0	0.87	0.80	0.97	1.07	0.80	0.139	0.794	0.729	0.731	0.125
d 0 to 14	0.67	0.69	0.83	1.01	0.80	0.141	0.286	0.772	0.307	0.218
d 0 to 28	0.70	0.75	0.75	0.86	0.83	0.110	0.401	0.898	0.388	0.898
d 0 to 42	0.78	0.76	0.74	0.90	0.76	0.092	0.891	0.398	0.347	0.508
d 0 to 56	0.65	0.69	0.68	0.74	0.55	0.061	0.865	0.125	0.545	0.139

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]NCTRL, U, NIT, UB, and NITB had 5, 5, 5, 5, and 4 experimental units, respectively; largest SEM was provided.
[6]Initial BW was the average of BW recorded on d -28 and -27.
[7]Final BW was the average of BW recorded on d 55 and 56.

Table 5-3. Effect of BSS[1] and eCAN[2] on carcass ultrasound measurements

	Treatment[3]					*P*-value[4]			
Item[6]	NCTRL	U	NIT	UB	NITB	SEM[5]	NPN	NS	B	B×N
LM area, cm^2	54.36	56.54	54.41	58.07	56.79	2.677	0.441	0.493	0.431	0.871
LM area change[7], cm^2	10.26	12.33	11.38	15.12	12.32	2.221	0.298	0.395	0.397	0.671
12th–rib fat thickness, cm	0.50	0.42	0.47	0.56	0.40	0.025	0.204	0.012	0.080	<0.001
12th–rib fat thickness change[8], cm	0.063	-0.025	0.025	0.117	-0.045	0.019	0.042	0.009	0.069	<0.001

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]NCTRL, U, NIT, UB, and NITB had 5, 5, 5, 5, and 4 experimental units, respectively; largest SEM was provided.
[6]LM area and 12th–rib fat thickness was measured on d 56 using d -28 as a covariate
[7]Difference in LM area between d -28 and 56.
[8]Difference in 12th–rib fat thickness between d -28 and 56.

Table 5-4. Effect of BSS[1] and eCAN[2] on liver mineral concentration

		Treatment[3]					*P*-value[4]			
Item	NCTRL	U	NIT	UB	NITB	SEM[5]	NPN	NS	B	B×N
Final liver mineral[6], mg/kg DM										
Cu	54.98	55.69	53.93	11.35	21.66	9.771	0.088	0.666	0.002	0.533
Fe	189.33	213.63	235.73	268.00	318.15	24.274	0.013	0.116	0.016	0.527
Zn	123.56	128.83	134.93	137.20	131.60	4.983	0.087	0.958	0.604	0.251
Mn	11.14	12.56	12.34	13.00	13.45	0.487	0.006	0.815	0.134	0.475
Se	0.33	0.38	0.37	0.31	0.33	0.035	0.613	0.921	0.125	0.637

[1]BSS = bismuth subsalicylate.
[2]eCAN = encapsulated calcium-ammonium nitrate ($5Ca(NO_3)_2$-NH_4NO_3) – 65.1% nitrate DM basis
[3]NCTRL = ad libitum bahiagrass hay + 2.7 g/kg of BW of molasses (basal diet); U = basal diet + 182 mg/kg of BW of urea; NIT = basal diet + 350 mg/kg of BW of NO_3^-; UB = U + 58.4 mg/kg of BW of BSS; NITB = NIT + 58.4 mg/kg of BW of BSS.
[4]Observed significance levels for: NPN = effect of NPN, NCTRL vs the mean of U + NIT + UB + NITB; NS = effect of NPN source (excludes NCTRL); B = effect of BSS (excludes NCTRL); B×N = interaction of BSS and eCAN.
[5]NCTRL, U, NIT, UB, and NITB had 5, 5, 5, 5, and 4 experimental units, respectively; largest SEM was provided.
[6]Liver mineral analyzed on d 56 using d -28 as a covariate.

CHAPTER 6
SUMMARY AND RECOMMENDATIONS

Summary

This series of experiments was designed to evaluate the effects of calcium-ammonium nitrate (**CAN**) and bismuth subsalicylate (**BSS**), a novel feed additive for ruminants, on beef cattle performance. To the best of the author's knowledge, this is the first work conducted in which ruminants received BSS in vivo. Much of the published research on BSS focuses on monogastrics, more specifically human and rat models, and gut health. One research group from the University of Minnesota included BSS in the substrate used for in vitro batch and continuous culture fermentations, hypothesizing that BSS would inhibit production of H_2S. In fact, H_2S was reduced by 34 and 99% when provided at 1% of the substrate DM with in vitro batch and continuous cultures, respectively. Furthermore, BSS did not appear to inhibit fermentation using the batch culture system when provided up to 1% of the substrate DM.

Providing a NPN source to ruminants is not a novel idea within the confines of protein and fiber production (Owen et al., 1942). Urea has been provided to cattle for years as an inexpensive source of N that ruminal microorganisms can convert to true protein. In recent years, nitrate has been investigated as a potential strategy to reduce the production of enteric CH_4 from ruminants by acting as a H_2 sink (Leng, 2008; van Zijderveld et al., 2010; Hulshof et al., 2012). It has been proposed that nitrate could also act as a NPN source for ruminal microorganisms through the same pathway as the proposed mode of action for the mitigation of CH_4, capturing H_2 and reducing nitrate to NH_3 (Leng, 2008; Newbold et al., 2014). A majority of the research published evaluating the CH_4 mitigating effects and potential role as a NPN source has focused on cattle

consuming at least 50% concentrate diets (Nolan et al., 2010; Newbold et al., 2014; Lee et al., 2017a); therefore, the current series of experiments was designed to evaluate nitrate supplementation to cattle consuming forage-based diets.

In the current series of experiments, 3 objectives were developed to test the hypothesis that CAN and BSS would improve beef cattle production while lessening environmental impacts. The objectives were: 1) to evaluate the effect of increasing doses of BSS in combination with CAN on in vitro ruminal fermentation; 2) Determine the effects of BSS and encapsulated CAN (**eCAN**) on in vivo ruminal fermentation, metabolism, nutrient digestibility, and enteric CH_4 production of beef cattle consuming a bahiagrass hay diet with supplemental molasses; and 3) Asses the effects of BSS in combination with eCAN on performance of growing cattle consuming a bahiagrass hay diet with supplemental molasses.

The first objective involved a series of 3 in vitro ruminal fermentation experiments. The first in vitro experiment evaluated CAN on fermentation, the second in vitro experiment considered the effects of CAN and BSS on fermentation parameters, and the third experiment was designed to determine a dose of BSS that would allow for reduction in H_2S production without having negative effects on other fermentation parameters. These experiments provided data that implied that CAN has the ability to effectively reduce in vitro CH_4 production; however, negative effects on in vitro OM digestibility (**IVOMD**), concentrations of NH_3-N, and gas production were also observed. There were few interactions between CAN and BSS when evaluated in vitro, with the exception of CH_4 production. When BSS was provided along with CAN, in place of urea,

in vitro CH_4 production (mmol/g of fermented OM) was decreased in an additive manner.

The production of H_2S in vitro was reduced by 61% when BSS was provided at 0.33% of the substrate DM. When included at 1.0% of the substrate DM, BSS had detrimental effects on fermentation, with reductions of 11, 29, and 23% for production of total VFA, IVOMD, and maximal gas production, respectively. In the third in vitro experiment, when BSS was provided alone at lesser concentrations, it was observed that at up 0.33% of the substrate DM, BSS reduced production of H_2S without impeding fermentation in vitro.

The second objective was composed of 2 in vivo experiments: 1) an experiment using 10 ruminally cannulated cattle to evaluate the effects of eCAN and BSS on ruminal metabolism and microbial N flow out of the rumen; and 2) an experiment using 25 crossbred steers to investigate the impacts of eCAN and BSS on enteric CH_4 production and total tract digestibility of nutrients. The metabolism experiment provided data that implied that eCAN altered ruminal fermentation by decreasing the production of total VFA. There were no differences observed between cattle that received urea or eCAN as NPN sources for microbial N flow; furthermore, no differences were observed between cattle that received a NPN source and those that did not, for microbial N flow. There was no observed impact of BSS on ruminal metabolism; however, rectal temperature was significantly reduced in cannulated steers and heifers that were receiving BSS. The explanation of this result is still unknown, but it allows for the opportunity of future research into the effects of BSS on inflammation.

The experiment designed to evaluate the effects of eCAN and BSS on total tract digestibility of nutrients and enteric CH_4 production provided results that support the data gained in the metabolism experiment. There was no impact of BSS on enteric CH_4 production nor nutrient digestibility; however, apparent retention of S was decreased with the addition of BSS to a bahiagrass hay diet supplemented with molasses. Enteric CH_4 production was decreased (g/d and g/kg of $BW^{0.75}$) with the addition of eCAN to the diet of steers. Total tract digestibility of nutrients (DM, OM, NDF, ADF, and CP) was also reduced with the addition of eCAN. Considering the reduction in digestibility, when CH_4 production was expressed as grams per kilogram of OM digested, there were no observable differences between cattle receiving urea and eCAN.

Finally, the third objective included a performance experiment which was composed of 75 growing heifers housed in 25 dormant, bahiagrass pastures. Data from this experiment implied that eCAN and BSS, alone, did not impact performance of growing cattle consuming forage-based diets; however, when provided in combination, a NPN source × BSS interaction was observed where final BW of heifers consuming eCAN and BSS appeared to be negatively affected. Furthermore, those heifers consuming both BSS and eCAN had lesser back fat thickness.

It was hypothesized that BSS would increase concentrations of trace minerals in the liver of heifers; however, data gained from this experiment indicated that concentrations of Cu in the liver were nearly depleted with the addition of BSS in the diet of growing heifers. This was accompanied by an increase in concentrations of liver Fe.

Recommendations

The data collected from the experiments described here within create a solid foundation for future research involving ruminants consuming forage-based diets. Not only did the data regarding the use of BSS and eCAN answer several pressing questions, but it also opened the door for researchers to pursue different avenues of research with nitrate and bismuth subsalicylate.

Calcium-Ammonium Nitrate

It appears from the data gained in this series of experiments, and that from other researchers, that eCAN may inhibit ruminal degradation of fiber due to inhibitory effects on cellulolytic bacteria (Asanuma et al., 2015; Latham et al., 2016). This likely explains why nitrate has been successfully used in feedlot and dairy diets to reduce enteric CH_4 production while either maintaining performance (Olijhoek et al., 2016) or improving feed efficiency (Newbold et al., 2014). After evaluating the data, it is not recommended to use eCAN as a NPN for cattle consuming low-quality, hay-based diets. It is possible that the high concentration of fiber in the hay is not being degraded as rapidly or extensively as it would be when urea is used as a NPN source. With concentrate-based diets, the limited amount of fiber may not be digested as well, but as it is a minor fraction of the diet, it may not affect animal performance. Future research should focus on the use of eCAN on differing types of forage and conservation methods (fresh vs. haylage or hay).

Another potential for eCAN in ruminant diets is to mitigate negative effects of high-S, feedlot diets, more specifically H_2S. Although the experiments described within this dissertation did not fully elucidate the impact of nitrate on production of H_2S, there is a strong potential for mitigating effects when Gibb's free energy for change is

considered. The ΔG°’ for the reduction of nitrate is -143.4 kcal while the reduction of sulfate has a ΔG°’ of -36.4 kcal (the more negative ΔG°’ indicates a more thermodynamically favorable reaction; Weimer, 1998). With the reduction of nitrate being more thermodynamically favorable than the reduction of sulfate, which is more thermodynamically favorable than methanogenesis (-36.4 vs. -33.2 kcal), it is probable that nitrate could be used as a dual purpose additive with multiple outcomes: NPN source and hydrogen sink with negative effects on methanogenesis and production of H_2S.

Bismuth Subsalicylate

It was hypothesized, prior to the commencement of this study, that BSS would have benefits on 2 fronts of beef cattle production: 1) reduce production of H_2S; and 2) mitigate negative effects of S on trace mineral concentration in the liver. This series of experiments did not fully define the potential of BSS to act as a limiter of production of H_2S, mainly due to the forage content of the diet provided to the cattle; however, in the third experiment (digestibility and enteric CH_4 production), data indicated that more S was being excreted from steers receiving BSS compared with those not receiving BSS. It would be beneficial to the field of ruminant nutrition to determine the impact of BSS on cattle receiving a high-S, high-concentrate diet.

Data from the performance experiment implied that BSS did not improve trace mineral accumulation in the liver of growing heifers, in fact, liver Cu was nearly depleted after 70 d of receiving BSS. This indicates that BSS should not be used by beef producers to improve trace mineral absorption when cattle are consuming high-S diets; however, there is a potential for BSS in another ruminant species: sheep. It has been recognized for decades that sheep are susceptible to disease and death caused by over

accumulation of Cu in the liver (Goodrich and Tillman, 1966). With the data observed in the performance experiment of this study, it could be speculated that BSS, when fed at concentrations that do not affect ruminal fermentation, could provide feed producers another avenue to combat Cu toxicity in sheep.

LIST OF REFERENCES

Ahvenjärvi, S., a Vanhatalo, P. Huhtanen, and T. Varvikko. 2000. Determination of reticulo-rumen and whole-stomach digestion in lactating cows by omasal canal or duodenal sampling. Br. J. Nutr. 83:67–77. doi:10.1017/S0007114500000106.

Appuhamy, J. A. D. R. N., A. B. Strathe, S. Jayasundara, C. Wagner-Riddle, J. Dijkstra, J. France, and E. Kebreab. 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. J. Dairy Sci. 96:5161–5173. doi:10.3168/jds.2012-5923. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23769353

Arthington, J. D., and L. R. Corah. 1995. Liver biopsy procedures for determining the trace mineral status in beef cows. Part II.

Arthington, J. D., J. E. Rechcigl, G. P. Yost, L. R. Mcdowell, and M. D. Fanning. 2002. Effect of ammonium sulfate fertilization on bahiagrass quality and copper metabolism in grazing beef cattle. J. Anim. Sci. 80:2507–2512.

Asanuma, N., S. Yokoyama, and T. Hino. 2015. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to Selenomonas ruminantium having the ability to reduce nitrate and nitrite. Anim. Sci. J. 86:378–384. doi:10.1111/asj.12307.

Ashes, J. R., S. K. Gulati, and T. W. Scott. 1997. Potential to alter the content and composition of milk fat through nutrition. J. Dairy Sci. 80:2204–2212. doi:10.3168/jds.S0022-0302(97)76169-1. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(97)76169-1

Balch, D. A., and S. J. Rowland. 1957. Volatile fatty acids and lactic acid in the rumen of dairy cows receiving a variety of diets. Br. Journalof Nutr. 11:288–298.

Barry, T. N., A. Thompson, and D. Armstrong. 1977. Rumen fermentation studies on two contrasting diets. 1. Some characteristics of the in vivo fermentation, with special reference to the composition of the gas phase, oxidation/reduction state and volatile fatty acid proportions. J. Agric. Sci. 89:183–195.

Bartley, E. E., E. L. Herod, R. M. Bechtle, D. A. Sapienza, and B. E. Brent. 1979. Effect of monensin or lasalocid, with and without niacin or amicloral, on rumen fermentation and feed efficiency. J. Anim. Sci. 49:1066–1075.

Beauchemin, K. A., H. Henry Janzen, S. M. Little, T. A. McAllister, and S. M. McGinn. 2010. Life cycle assessment of greenhouse gas emissions from beef production in western Canada: A case study. Agric. Syst. 103:371–379. doi:10.1016/j.agsy.2010.03.008. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0308521X10000387

Beauchemin, K. A., H. H. Janzen, S. M. Little, T. A. McAllister, and S. M. McGinn. 2011.

Mitigation of greenhouse gas emissions from beef production in western Canada – Evaluation using farm-based life cycle assessment. Anim. Feed Sci. Technol. 166–167:663–677. doi:10.1016/j.anifeedsci.2011.04.047. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0377840111001660

Beauchemin, K. A., S. M. McGinn, T. F. Martinez, and T. A. McAllister. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85:1990–6. doi:10.2527/jas.2006-686. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17468433

Benu, I., M. J. Callaghan, N. Tomkins, G. Hepworth, L. A. Fitzpatrick, and A. J. Parker. 2016. The effect of feeding frequency and dose rate of nitrate supplements on blood haemoglobin fractions in Bos indicus cattle fed Flinders grass (Iseilemia spp.) hay. Anim. Prod. Sci. 56:1605–1611. doi:10.1071/AN14886.

Binnerts, W. T., A. T. Van't Klooster, and A. M. Frens. 1968. Soluble chromium indicator measured by atomic absorption in digestion experiments. Vet. Rec. 82:470.

Bland, M. V., S. Ismail, J. A. Heinemann, and J. I. Keenan. 2004. The action of bismuth against Helicobacter pylori mimics but is not caused by intracellular iron deprivation. Antimicrob. Agents Chemother. 48:1983–1988. doi:10.1128/AAC.48.6.1983-1988.2004.

Boadi, D., C. Benchaar, J. Chiquette, and D. Massé. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84:319–335. doi:10.4141/A03-109. Available from: http://pubs.aic.ca/doi/abs/10.4141/A03-109

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

Bradley, A. S., W. D. Leavitt, and D. T. Johnston. 2011. Revisiting the dissimilatory sulfate reduction pathway. Geobiology. 9:446–457. doi:10.1111/j.1472-4669.2011.00292.x.

Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63:64.

Buddle, B. M., M. Denis, G. T. Attwood, E. Altermann, P. H. Janssen, R. S. Ronimus, C. S. Pinares-Patiño, S. Muetzel, and D. Neil Wedlock. 2011. Strategies to reduce methane emissions from farmed ruminants grazing on pasture. Vet. J. 188:11–17. doi:10.1016/j.tvjl.2010.02.019. Available from: http://dx.doi.org/10.1016/j.tvjl.2010.02.019

Butler, A. R., and M. Feelisch. 2008. Therapeutic uses of inorganic nitrite and nitrate: From the past to the future. Circulation. 117:2151–2159.

doi:10.1161/CIRCULATIONAHA.107.753814.

Cammack, K. M., C. L. Wright, K. J. Austin, P. S. Johnson, R. R. Cockrum, K. L. Kessler, and K. C. Olson. 2010. Effects of high-sulfur water and clinoptilolite on health and growth performance of steers fed forage-based diets. J. Anim. Sci. 88:1777–1785. doi:10.2527/jas.2009-2343.

Cashman, J. N. 1996. The Mechanisms of Action of NSAIDs in Analgesia. Drugs. 52:13–23. doi:10.2165/00003495-199600525-00004. Available from: http://link.springer.com/10.2165/00003495-199600525-00004

Chung, Y., Y. Su, C. Chen, G. Jia, H. Wang, J. C. G. Wu, and J. Lin. 2004. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacol. Sin. 25:932–936. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15210068

Ciriaco, F. M., D. D. Henry, V. R. G. Mercadante, T. M. Schulmeister, M. Ruiz-Moreno, G. C. Lamb, and N. DiLorenzo. 2016. Effects of molasses and crude glycerol combined in a liquid supplement on ruminal fermentation in beef steers consuming bermudagrass hay. J. Anim. Sci. 94:3851–3863. doi:10.2527/jas2016-0491.

Cole, N. A., K. McCuistion, L. W. Greene, and F. T. McCollum. 2011. Effects of concentration and source of wet distillers grains on digestibility of steam-flaked corn-based diets fed to finishing steers1. Prof. Anim. Sci. 27:302–311. doi:10.15232/S1080-7446(15)30493-9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1080744615304939

Counotte, G. H. M., A. T. van't Klooster, J. van der Kuilen, and R. A. Prins. 1979. An Analysis of the Buffer System in the Rumen of Dairy Cattle. J. Anim. Sci. 49:1536–1544.

Daetz, R., F. Cunha, J. H. Bittar, C. A. Risco, F. Magalhaes, Y. Maeda, J. E. P. Santos, K. C. Jeong, R. F. Cooke, and K. N. Galvão. 2016. Clinical response after chitosan microparticle administration and preliminary assessment of efficacy in preventing metritis in lactating dairy cows. J. Dairy Sci. 99:8946–8955. doi:10.3168/jds.2016-11400. Available from: http://dx.doi.org/10.3168/jds.2016-11400

Dewhurst, R. J., D. R. Davies, and R. J. Merry. 2000. Microbial protein supply from the rumen. Anim. Feed Sci. Technol. 85:1–21. doi:10.1016/S0377-8401(00)00139-5.

Dickhoefer, U., S. Ahnert, and A. Susenbeth. 2016. Effects of quebracho tannin extract on rumen fermentation and yield and composition of microbial mass in heifers. J. Anim. Sci. 94:1561–1575. doi:10.2527/jas2015-0061.

DiLorenzo, N., and M. L. Galyean. 2010. Applying technology with newer feed ingredients in feedlot diets: do the old paradigms apply? J. Anim. Sci. 88:E123–

E132. doi:10.2527/jas.2009-2362.

Domenico, P., J. Reich, W. Madonia, and B. A. Cunha. 1996. Resistance to bismuth among Gram-negative bacteria is dependent upon iron and its uptake. J. Antimicrob. Chemother. 38:1031–1040. doi:10.1093/jac/38.6.1031.

Domescik, E. J., and S. A. Martin. 1999. Effects of laidlomycin propionate and monensin on the in vitro mixed ruminal microorganism fermentation. J. Anim. Sci. 77:2305–2312.

Doreau, M., H. M. G. van der Werf, D. Micol, H. Dubroeucq, J. Agabriel, Y. Rochette, and C. Martin. 2011. Enteric methane production and greenhouse gases balance of diets differing in concentrate in the fattening phase of a beef production system. J. Anim. Sci. 89:2518–2528. doi:10.2527/jas.2010-3140. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21383032

Drewnoski, M. E., D. J. Pogge, and S. L. Hansen. 2014. High-sulfur in beef cattle diets : A review. J. Anim. Sci. 92:3763–3780. doi:10.2527/jas2013-7242.

Drewnoski, M. E., E. L. Richter, and S. L. Hansen. 2012. Dietary sulfur concentration affects rumen hydrogen sulfide concentrations in feed-lot steers during transition and finishing. J. Anim. Sci. 90:4478–4486. doi:10.2527/jas.2012-5078.

Duffield, T. F., A. R. Rabiee, and I. J. Lean. 2008. A meta-analysis of the impact of monensin in lactating dairy cattle. Part 2. Production effects. J. Dairy Sci. 91:1347–1360. doi:10.3168/jds.2007-0608. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18349227

Duin, E. C., T. Wagner, S. Shima, D. Prakash, B. Cronin, D. R. Yáñez-Ruiz, S. Duval, R. Rümbeli, R. T. Stemmler, R. K. Thauer, and M. Kindermann. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proc. Natl. Acad. Sci. 113:6172–6177. doi:10.1073/pnas.1600298113. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1600298113

Ebert, P. J., E. A. Bailey, A. L. Shreck, J. S. Jennings, and N. A. Cole. 2017. Effect of condensed tannin extract supplementation on growth performance, nitrogen balance, gas emissions, and energetic losses of beef steers. J. Anim. Sci. 95:1345–1355. doi:10.2527/jas2016.0341.

El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. J. Anim. Sci. 92:2214–2224. doi:10.2527/jas2013-7084.

Ellis, J. L., J. Dijkstra, A. Bannink, E. Kebreab, S. E. Hook, S. Archibeque, and J. France. 2012. Quantifying the effect of monensin dose on the rumen volatile fatty acid profile in high-grain-fed beef cattle. J. Anim. Sci. 90:2717–2726.

doi:10.2527/jas.2011-3966. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22896736

EPA. 2006. Global Anthropogenic Non-CO2 Greenhouse Gas Emissions : 1990 - 2020. Washington, D.C.

Eugène, M., D. Masse, J. Chiquette, and C. Benchaar. 2008. Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. Can. J. Anim. Sci. 88:331–334.

FAO. 2013. Mitigation of greenhouse gas emissions in livestock production – A review of technical options for non-CO2 emissions. (P. J. Gerber, B. Henderson, and H. P. S. Makkar, editors.).

Felix, T. L., W. P. Weiss, F. L. Fluharty, and S. C. Loerch. 2012. Effects of copper supplementation on feedlot performance, carcass characteristics, and rumen sulfur metabolism of growing cattle fed diets containing 60% dried distillers grains. J. Anim. Sci. 90:2710–2716. doi:10.2527/jas2011-4100.

Fiorentini, G., I. P. C. Carvalho, J. D. Messana, P. S. Castagnino, A. Berndt, R. C. Canesin, R. T. S. Frighetto, and T. T. Berchielli. 2014. Effect of lipid sources with different fatty acid profiles on the intake, performance, and methane emissions of feedlot Nellore steers. J. Anim. Sci. 92:1613–1620. doi:10.2527/jas2013-6868.

Goodrich, R. D., J. E. Garrett, D. R. Gast, M. A. Kirick, D. A. Larson, and J. C. Meiske. 1984. Influence of Monensin on the Performance of Cattle. J. Anim. Sci. 58:1484–1498.

Goodrich, R. D., and A. D. Tillman. 1966. Effects of sulfur and nitrogen sources and copper levels on the metabolism of certain minerals by sheep. J. Anim. Sci. 25:484–491.

Gould, D. H. 1998. Polioencephalomalacia. J. Anim. Sci. 76:309–314.

Gozho, G. N., J. J. McKinnon, D. A. Christensen, V. Racz, and T. Mutsvangwa. 2009. Effects of type of canola protein supplement on ruminal fermentation and nutrient flow to the duodenum in beef heifers. J. Anim. Sci. 87:3363–3371. doi:10.2527/jas.2009-1841.

Grainger, C., T. Clarke, M. J. Auldist, K. A. Beauchemin, S. M. Mcginn, G. C. Waghorn, and R. J. Eckard. 2009. Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 241–251.

Gregorini, P., S. A. Gunter, and P. A. Beck. 2008. Matching plant and animal processes to alter nutrient supply in strip-grazed cattle: Timing of herbage and fasting allocation. J. Anim. Sci. 86:1006–1020. doi:10.2527/jas.2007-0432.

Guan, H., K. M. Wittenberg, K. H. Ominski, and D. O. Krause. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. J. Anim. Sci. 84:1896–1906. doi:10.2527/jas.2005-652.

Guyader, J., M. Eugène, B. Meunier, M. Doreau, D. P. Morgavi, M. Silberberg, Y. Rochette, C. Gerard, C. Loncke, and C. Martin. 2015. Additive methane-mitigating effect between linseed oil and nitrate fed to cattle. J. Anim. Sci. 93:3564–3577. doi:10.2527/jas.2014-8196.

Guyader, J., M. Tavendale, C. Martin, and S. Muetzel. 2016. Dose-response effect of nitrate on hydrogen distribution between rumen fermentation end products: An in vitro approach. Anim. Prod. Sci. 56:224–230. doi:10.1071/AN15526.

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

Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119. doi:10.3168/jds.2013-7834. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24630651

Hansen, S. L., N. Trakooljul, H. C. S. Liu, J. A. Hicks, M. S. Ashwell, and J. W. Spears. 2010. Proteins involved in iron metabolism in beef cattle are affected by copper deficiency in combination with high dietary manganese, but not by copper deficiency alone. J. Anim. Sci. 88:275–283. doi:10.2527/jas.2009-1846.

Harper, L. A., O. T. Denmead, J. R. Freney, and F. M. Byers. 1999. Direct measurements of methane emissions from grazing and feedlot cattle. J. Anim. Sci. 77:1392–1401.

Hawksworth, E. L., P. C. Andrews, W. Lie, B. Lai, and C. T. Dillon. 2014. Biological evaluation of bismuth non-steroidal anti-inflammatory drugs (BiNSAIDs): Stability, toxicity and uptake in HCT-8 colon cancer cells. J. Inorg. Biochem. 135:28–39. doi:10.1016/j.jinorgbio.2014.02.012. Available from: http://dx.doi.org/10.1016/j.jinorgbio.2014.02.012

Hegarty, R. S., J. Miller, N. Oelbrandt, L. Li, J. P. M. Luijben, D. L. Robinson, J. V Nolan, and H. B. Perdok. 2016. Feed intake, growth, and body and carcass attributes of feedlot steers supplemented with two levels of calcium nitrate or urea. J. Anim. Sci. 94:5372–5381. doi:10.2527/jas2015-0266.

Henry, D. D., M. Ruiz-Moreno, F. M. Ciriaco, M. Kohmann, V. R. G. Mercadante, G. C. Lamb, and N. DiLorenzo. 2015. Effects of chitosan on nutrient digestibility, methane emissions, and in vitro fermentation in beef cattle. J. Anim. Sci. 93:3539–3550. doi:10.2527/jas2014-8844.

Hino, T., and J. B. Russell. 1987. Relative contributions of ruminal bacteria and protozoa to the degradation of protein in vitro. J. Anim. Sci. 64:261–270.

Hook, S. E., A.-D. G. Wright, and B. W. McBride. 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea. 2010:945785. doi:10.1155/2010/945785. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3021854&tool=pmcentrez&rendertype=abstract

Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, S. M. van Zijderveld, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. J. Anim. Sci. 90:2317–2323. doi:10.2527/jas2011-4209.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160:1–22. doi:10.1016/j.anifeedsci.2010.07.002. Available from: http://dx.doi.org/10.1016/j.anifeedsci.2010.07.002

Jenkins, T. C., H. G. Bateman, and S. M. Block. 1996. Butylsoyamide increases unsaturation of fatty acids in plasma and milk of lactating dairy cows. J. Dairy Sci. 79:585–590. doi:10.3168/jds.S0022-0302(96)76403-2. Available from: http://dx.doi.org/10.3168/jds.S0022-0302(96)76403-2

Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8567486

Johnson, K., M. Huyler, H. Westberg, B. Lamb, and P. Zlmmerman. 1994. Measurement of methane emissions from ruminant livestock using a SF6 tracer technique. Environ. Sci. Technol. 28:359–362.

Klop, G., J. Dijkstra, K. Dieho, W. H. Hendriks, and A. Bannink. 2017. Enteric methane production in lactating dairy cows with continuous feeding of essential oils or rotational feeding of essential oils and lauric acid. J. Dairy Sci. 100:3563–3575. doi:https://doi.org/10.3168/jds.2016-12033. Available from: http://www.sciencedirect.com/science/article/pii/S0022030217301698%5Cnhttp://www.journalofdairyscience.org/article/S0022-0302(17)30169-8/fulltext

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2008. BOARD-INVITED REVIEW: Use of distillers by-products in the beef cattle feeding industry. J. Anim. Sci. 86:1223–1231. doi:10.2527/jas.2007-0550. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18156361

Kobayashi, K., S. Tachibana, and M. Ishimoto. 1969. Intermediary formation of trithionate in sulfite reduction by a sulfate-reducing bacterium. J. Biochem. 65:155–157.

Kristensen, T., L. Mogensen, M. T. Knudsen, and J. E. Hermansen. 2011. Effect of production system and farming strategy on greenhouse gas emissions from commercial dairy farms in a life cycle approach. Livest. Sci. 140:136–148. doi:10.1016/j.livsci.2011.03.002. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1871141311000850

Krizsan, S. J., and P. Huhtanen. 2013. Effect of diet composition and incubation time on feed indigestible neutral detergent fiber concentration in dairy cows. J. Dairy Sci. 96:1715–1726.

Latham, E. A., R. C. Anderson, W. E. Pinchak, and D. J. Nisbet. 2016. Insights on alterations to the rumen ecosystem by nitrate and nitrocompounds. Front. Microbiol. 7:1–15. doi:10.3389/fmicb.2016.00228.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015a. Effects of encapsulated nitrate on eating behavior, rumen fermentation, and blood profile of beef heifers fed restrictively or ad libitum. J. Anim. Sci. 93:2405–2418. doi:10.2527/jas2014-8851.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015b. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. J. Anim. Sci. 93:2391–2404. doi:10.2527/jas2014-8845.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2015c. Effects of feed consumption rate of beef cattle offered a diet supplemented with nitrate ad libitum or restrictively on potential toxicity of nitrate. J. Anim. Sci. 93:4956–4966. doi:10.2527/jas.2015-9435.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017a. Effects of encapsulated nitrate on growth performance, carcass characteristics, nitrate residues in tissues, and enteric methane emissions in beef steers: Finishing phase. J. Anim. Sci. 95:3712–3726. doi:10.2527/jas2017.1461.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017b. Effects of encapsulated nitrate on growth performance, nitrate toxicity, and enteric methane emissions in beef steers: Backgrounding phase. J. Anim. Sci. 95:3700–3711. doi:10.2527/jas2014-8845.

Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2017c. 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. Anim. Feed Sci. Technol. 231:97–106. doi:10.1016/j.anifeedsci.2017.07.005. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0377840117301839

Lee, C., and K. A. Beauchemin. 2014. A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. Can. J. Anim. Sci. 94:557–570. doi:10.4141/cjas-2014-069.

Available from: http://pubs.aic.ca/doi/abs/10.4141/cjas-2014-069

Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A Rep. to Dep. Clim. Chang. Commonw. Gov. Aust. Canberra. Available from: http://www.penambulbooks.com

Levine, J., C. J. Ellis, J. K. Furne, J. Springfield, and M. D. Levitt. 1998. Fecal hydrogen sulfide production in ulcerative colitis. Am. J. Gastroenterol. 93:83–87. doi:10.1111/j.1572-0241.1998.083_c.x.

Levitt, M. D., J. Springfield, J. Furne, T. Koenig, and F. L. Suarez. 2002. Physiology of sulfide in the rat colon: use of bismuth to assess colonic sulfide production. J. Appl. Physiol. 92:1655–1660. doi:10.1152/japplphysiol.00907.2001.

Li, L., J. Davis, J. Nolan, and R. Hegarty. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. Anim. Prod. Sci. 52:653–658. doi:10.1071/AN11254.

Li, L., C. I. Silveira, J. V. Nolan, I. R. Godwin, R. A. Leng, and R. S. Hegarty. 2013. Effect of added dietary nitrate and elemental sulfur on wool growth and methane emission of Merino lambs. Anim. Prod. Sci. 53:1195–1201. doi:10.1071/AN13222.

Lin, M., D. M. Schaefer, G. Q. Zhao, and Q. X. Meng. 2013. Effects of nitrate adaptation by rumen inocula donors and substrate fiber proportion on in vitro nitrate disappearance, methanogenesis, and rumen fermentation acid. Animal. 7:1099–1105. doi:10.1017/S1751731113000116. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23391259

Linneen, S. K., A. R. Harding, M. T. Smallwood, G. W. Horn, J. S. Jennings, C. L. Goad, and D. L. Lalman. 2015. In vivo ruminal degradation characteristics and apparent digestibility of low-quality prairie hay for steers consuming monensin and Optimase. J. Anim. Sci. 93:3941–3949. doi:10.2527/jas2014-8772.

Lund, P., R. Dahl, H. J. Yang, A. L. F. Hellwing, B. B. Cao, and M. R. Weisbjerg. 2014. The acute effect of addition of nitrate on in vitro and in vivo methane emission in dairy cows. Anim. Prod. Sci. 54:1432–1435. doi:10.1071/AN14339.

Manhart, M. D. 1990. In vitro antimicrobial activity of bismuth subsalicylate and other bismuth salts. Rev. Infect. Dis. 12 Suppl 1:S11–S15.

Marais, J. P. 1988. Effect of nitrate and its reduction products on the growth and activity of the rumen microbial population. Br. Journalof Nutr. 59:301–313.

Martínez-Fernández, G., L. Abecia, A. Arco, G. Cantalapiedra-Hijar, a I. Martín-García, E. Molina-Alcaide, M. Kindermann, S. Duval, and D. R. Yáñez-Ruiz. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal

fermentation, microbial abundance, and methane emissions in sheep. J. Dairy Sci. 97:3790–3799. doi:10.3168/jds.2013-7398. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24731636

Martínez, M. E., M. J. Ranilla, S. Ramos, M. L. Tejido, C. Saro, and M. D. Carro. 2009. Evaluation of procedures for detaching particle-associated microbes from forage and concentrate incubated in Rusitec fermenters: efficiency of recovery and representativeness of microbial isolates. J. Anim. Sci. 87:2064–2072. doi:10.2527/jas.2008-1634.

Mc Geough, E. J., P. O'Kiely, K. J. Hart, a P. Moloney, T. M. Boland, and D. a Kenny. 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. J. Anim. Sci. 88:2703–2716. doi:10.2527/jas.2009-2750. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20382872

McDougall, E. I. 1944. Studies on ruminant saliva: The composition and output of sheep's saliva. Biochem. J. 43:99–109.

McMahon, L. R., T. A. McAllister, B. P. Berg, W. Majak, S. N. Acharya, J. D. Popp, B. E. Coulman, Y. Wang, and K.-J. Cheng. 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Can. J. Plant Sci. 80:469–485. doi:10.4141/P99-050. Available from: http://pubs.aic.ca/doi/abs/10.4141/P99-050

Mills, C. F., A. C. Dalgarno, and G. Wenham. 1976. Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. Br. J. Nutr. 35:309–331. doi:10.1079/BJN19760039. Available from: http://www.ncbi.nlm.nih.gov/pubmed/178348

Mitsui, T., L. M. Edmond, E. a. Magee, and J. H. Cummings. 2003. The effects of bismuth, iron, zinc and nitrate on free sulfide in batch cultures seeded with fecal flora. Clin. Chim. Acta. 335:131–135. doi:10.1016/S0009-8981(03)00288-2. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0009898103002882

Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal. 6:871. doi:10.1017/S1751731112000407.

Morine, S. J., M. E. Drewnoski, and S. L. Hansen. 2014a. Increasing dietary neutral detergent fiber concentration decreases ruminal hydrogen sulfide concentrations in steers fed high-sulfur diets based on ethanol coproducts. J. Anim. Feed Sci. 92:3035–3041. doi:10.2527/jas2013-7339.

Morine, S. J., M. E. Drewnoski, A. K. Johnson, and S. L. Hansen. 2014b. Determining the influence of dietary roughage concentration and source on ruminal parameters related to sulfur toxicity. J. Anim. Sci. 92:4068–4076. doi:10.2527/jas2013-6925.

NASEM. 2016. Nutrient Requirments of Beef Cattle. 8th Revise. The National Academies Press, Washington, D.C.

Van Nevel, C. J., and D. I. Demeyer. 1996. Control of rumen methanogenesis. Environ. Monit. Assess. 42:73–97. doi:10.1007/BF00394043.

Newbold, J. R., S. M. van Zijderveld, R. B. A. Hulshof, W. B. Fokkink, R. A. Leng, P. Terenecio, W. J. Powers, P. S. J. Van Adrichem, N. D. Paton, and H. B. Perdok. 2014. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. J. Anim. Sci. 92:5032–5040. doi:10.2527/jas2014-7677.

Nguyen, S. H., M. C. Barnett, and R. S. Hegarty. 2016. Use of dietary nitrate to increase productivity and reduce methane production of defaunated and faunated lambs consuming protein-deficient chaff. Anim. Prod. Sci. 56:290–297. doi:10.1071/AN15525.

Nichols, C. A., V. R. Bremer, A. K. Watson, C. D. Buckner, J. L. Harding, D. R. Smith, G. E. Erickson, and T. J. Klopfenstein. 2012. Meta-Analysis of the Effect of Dietary Sulfur on Feedlot Health.

Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. Anim. Prod. Sci. 50:801–806. doi:10.1071/AN09211. Available from: http://www.publish.csiro.au/?paper=AN09211

Olijhoek, D. W., A. L. F. Hellwing, M. Brask, M. R. Weisbjerg, O. Højberg, M. K. Larsen, J. Dijkstra, E. J. Erlandsen, and P. Lund. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. J. Dairy Sci. 99:6191–6205. doi:10.3168/jds.2015-10691. Available from: http://dx.doi.org/10.3168/jds.2015-10691

Owen, E. C., J. A. B. Smith, and N. C. Wright. 1942. Urea as a Partial Protein Substitute in the Feeding of Dairy Cattle. Biochem. J. 37:44–53.

Owens, F. N., D. S. Secrist, W. J. Hill, and D. R. Gill. 1998. Acidosis in cattle: a review. J. Anim. Sci. 76:275–286.

Ozturk, A. S., M. Guzel, T. K. Askar, and I. Aytekin. 2013. Evaluation of the hormones responsible for the gastrointestinal motility in cattle with displacement of the abomasum; ghrelin, motilin and gastrin. Vet. Rec. 172:636.

Pannequin, J., S. Kovac, J. P. Tantiongco, R. S. Norton, A. Shulkes, K. J. Barnham, and G. S. Baldwin. 2004. A novel effect of bismuth ions: Selective inhibition of the biological activity of glycine-extended gastrin. J. Biol. Chem. 279:2453–2460. doi:10.1074/jbc.M309806200.

Pellikaan, W. F., E. Stringano, J. Leenaars, D. J. G. M. Bongers, S. van L. van Schuppen, J. Plant, and I. Mueller-Harvey. 2011. Evaluating effects of tannins on extent and rate of in vitro gas and CH4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166–167:377–390. doi:10.1016/j.anifeedsci.2011.04.072. Available from: http://dx.doi.org/10.1016/j.anifeedsci.2011.04.072

Phillips, H. a., M. D. Eelman, and N. Burford. 2007. Cooperative influence of thiolate ligands on the bio-relevant coordination chemistry of bismuth. J. Inorg. Biochem. 101:736–739. doi:10.1016/j.jinorgbio.2007.01.003.

Pitcher, M. C., E. R. Beatty, and J. H. Cummings. 2000. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. Gut. 46:64–72. doi:10.1136/gut.46.1.64.

Pogge, D. J., M. E. Drewnoski, and S. L. Hansen. 2014. High dietary sulfur decreases the retention of copper, manganese, and zinc in steers. J. Anim. Sci. 92:2182–2191. doi:10.2527/jas2013-7481.

Pogge, D. J., and S. L. Hansen. 2013. Supplemental vitamin C improves marbling in feedlot cattle consuming high sulfur diets. J. Anim. Sci. 91:4303–4314. doi:10.2527/jas2012-5638.

Prins, R. A., C. J. Van Nevel, and D. I. Demeyer. 1972. Pure culture studies of inhibitors for methanogenic bacteria. Antonie Van Leeuwenhoek. 38:281–287. doi:10.1007/BF02328099.

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

Qi, L., Z. Xu, X. Jiang, C. Hu, and X. Zou. 2004. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr. Res. 339:2693–2700. doi:10.1016/j.carres.2004.09.007. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15519328

de Raphélis-Soissan, V., L. Li, I. R. Godwin, M. C. Barnett, H. B. Perdok, and R. S. Hegarty. 2014. Use of nitrate and Propionibacterium acidipropionici to reduce methane emissions and increase wool growth of Merino sheep. Anim. Prod. Sci. 54:1860–1866. doi:10.1071/AN14329.

Rees, C. E. 1973. A steady-state model for sulphur isotope fractionation in bacterial reduction processes. Geochim. Cosmochim. Acta. 37:1141–1162.

Reynolds, C. K., D. J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 97:3777–89. doi:10.3168/jds.2013-7397. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/24704240

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

Romero-Perez, A., E. K. Okine, S. M. McGinn, L. L. Guan, M. Oba, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. J. Anim. Sci. 92:4682–4693. doi:10.2527/jas2014-7573.

Romero, J. J., M. A. Zarate, O. C. M. Queiroz, J. H. Han, J. H. Shin, C. R. Staples, W. F. Brown, and A. T. Adesogan. 2013. Fibrolytic enzyme and ammonia application effects on the nutritive value, intake, and digestion kinetics of bermudagrass hay in beef cattle. J. Anim. Sci. 91:4345–4356. doi:10.2527/jas2013-6261.

Ruiz-Moreno, M., E. Binversie, S. W. Fessenden, and M. D. Stern. 2015. Mitigation of in vitro hydrogen sulfide production using bismuth subsalicylate with and without monensin in beef feedlot diets. J. Anim. Sci. 93:5346–5354. doi:10.2527/jas2015-9392.

Russell, R. W., and S. A. Gahr. 2000. Glucose availability and associated metabolism. In: Farm Animal Metabolism and Nutrition. p. 121–147.

Sar, C., B. Mwenya, B. Santoso, K. Takaura, R. Morikawa, N. Isogai, Y. Asakura, Y. Toride, and J. Takahashi. 2005. Effect of Escherichia coli W3110 on ruminal methanogenesis and nitrate/nitrite reduction in vitro. Anim. Feed Sci. Technol. 118:295–306. doi:10.1016/j.anifeedsci.2004.10.004.

Sar, C., B. Santoso, B. Mwenya, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Manipulation of rumen methanogenesis by the combination of nitrate with β1-4 galacto-oligosaccharides or nisin in sheep. Anim. Feed Sci. Technol. 115:129–142. doi:10.1016/j.anifeedsci.2004.01.006.

Sarturi, J. O., G. E. Erickson, T. J. Klopfenstein, K. M. Rolfe, C. D. Buckner, and M. K. Luebbe. 2013. Impact of source of sulfur on ruminal hydrogen sulfide and logic for the ruminal available sulfur for reduction concept. J. Anim. Sci. 91:3352–3359. doi:10.2527/jas.2012-5626.

Satter, L. D., and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr. 32:199–208. doi:10.1079/BJN19740073. Available from: http://journals.cambridge.org/abstract_S0007114574000742

Shibata, M., F. Terada, K. Iwasaki, M. Kurihara, and T. Nishida. 1992. Methane Production in Heifers, Sheep and Goats Consuming Diets of Various Hay-Concentrate Ratios. Anim. Sci. Technol. 63:1221–1227.

Shike, D. W. 2013. Beef cattle feed efficiency. In: Driftless Region Beef Conference 2013. p. 3–5.

Smith, D. R., N. Dilorenzo, J. Leibovich, M. L. May, M. J. Quinn, J. W. Homm, and M. L. Galyean. 2010. Effects of sulfur and monensin concentrations on in vitro dry matter disappearance, hydrogen sulfide production, and volatile fatty acid concentrations in batch culture ruminal fermentations. J. Anim. Sci. 88:1503–1512. doi:10.2527/jas.2009-2498. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20023144

Van Soest, P. J. 1994. Nutritional ecology of the ruminant.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–97. doi:10.3168/jds.S0022-0302(91)78551-2. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1660498

Sox, T. E., and C. A. Olson. 1989. Binding and killing of bacteria by bismuth subsalicylate. Antimicrob. Agents Chemother. 33:2075–2082. doi:10.1128/AAC.33.12.2075.

Spears, J. W., K. E. Lloyd, and R. S. Fry. 2011. Tolerance of cattle to increased dietary sulfur and effect of dietary cation-anion balance. J. Anim. Sci. 89:2502–2509. doi:10.2527/jas.2010-3265.

Suarez, F. L., J. K. Furne, J. Springfield, and M. D. Levitt. 1998. Bismuth subsalicylate markedly decreases hydrogen sulfide release in the human colon. Gastroenterology. 923–929.

Sun, Y. K., X. G. Yan, Z. B. Ban, H. M. Yang, R. S. Hegarty, and Y. M. Zhao. 2017. The effect of cysteamine hydrochloride and nitrate supplementation on in-vitro and in-vivo methane production and productivity of cattle. Anim. Feed Sci. Technol. 232:49–56. doi:10.1016/j.anifeedsci.2017.03.016. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0377840116308628

Suttle, N. 2010. Mineral nutrition of livestock. 4th ed. CABI, Oxfordshire, UK.

Suttle, N. F. 1991. The interactions between copper, molybdenum, and sulfur in ruminant nutrition. Annu. Rev. Nutr. 11:121–140.

Szymanska, J. A., and A. J. Zelazowski. 1979. Effect of cadmium, mercury, and bismuth on the copper content in rat tissues. Environ. Res. 19:121–126.

Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. J. Br. Grassl. Soc. 18:104–111.

Tomkins, N., A. J. Parker, G. Hepworth, and M. J. Callaghan. 2016. Nitrate supplementation has marginal effects on enteric methane production from Bos

indicus steers fed Flinders grass (Iseilema spp .) hay , but elevates blood methaemoglobin concentrations. Anim. Prod. Sci. doi:10.1071/AN16002. Available from: http://dx.doi.org/10.1071/AN16002%0ANitrate

Truong, D. H., M. A. Eghbal, W. Hindmarsh, S. H. Roth, and P. J. O'Brien. 2006. Molecular mechanisms of hydrogen sulfide toxicity. Drug Metab. Rev. 38:733–744. doi:10.1080/03602530600959607. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17145698

Ungerfeld, E. M. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. Front. Microbiol. 6:1–17. doi:10.3389/fmicb.2015.00037.

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

USDA. 2004. US Agriculture and Forestry Greenhouse Gas Inventory: 1990-2001. 1907:1–164.

Uwituze, S., G. L. Parsons, C. J. Schneider, K. K. Karges, M. L. Gibson, L. C. Hollis, J. J. Higgins, and J. S. Drouillard. 2011. Evaluation of sulfur content of dried distillers grains with solubles in finishing diets based on steam-flaked corn or dry-rolled corn. J. Anim. Sci. 89:2582–2591. doi:10.2527/jas.2010-3103.

Velazco, J. I., D. J. Cottle, and R. S. Hegarty. 2014. Methane emissions and feeding behaviour of feedlot cattle supplemented with nitrate or urea. Anim. Prod. Sci. 54:1737–1740. doi:10.1071/AN14345.

Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, H. B. Perdok, and C. J. Newbold. 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One. 10:1–18. doi:10.1371/journal.pone.0140282.

Vyas, D., S. M. McGinn, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2016. Effects of sustained reduction of enteric methane emissions with dietary supplementation of 3-nitrooxypropanol on growth performance of growing and finishing beef cattle. J. Anim. Sci. 94:2024–2034. doi:10.2527/jas2015-0268.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Anim. Feed Sci. Technol. 147:116–139. doi:10.1016/j.anifeedsci.2007.09.013. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0377840107003616

Weeth, H. J., and D. L. Capps. 1972. Tolerance of growing cattle for sulfate-water. J. Anim. Sci. 34:256–260.

Weeth, H. J., and J. E. Hunter. 1971. Drinking of sulfate-water by cattle. J. Anim. Sci. 32:277–281.

Weimer, P. J. 1998. Manipulating ruminal fermentation: a microbial ecological perspective. J. Anim. Sci. 76:3114–3122. doi:10.1016/j.athoracsur.2007.03.023.

Yakoob, J., Z. Abbas, M. W. Usman, S. Awan, S. Naz, F. Jafri, S. Hamid, and W. Jafri. 2013. Comparison of Antimicrobial Activity of Zinc Chloride and Bismuth Subsalicylate Against Clinical Isolates of Helicobacter pylori. Microb Drug Resist. 20. doi:10.1089/mdr.2013.0086. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23844851

Yan, C., E. Gu, F. Liu, Y. Lai, J. Li, and Y. Liu. 2013. Colloidal synthesis and characterizations of wittichenite copper bismuth sulphide nanocrystals. Nanoscale. 5:1789. doi:10.1039/c3nr33268c. Available from: http://xlink.rsc.org/?DOI=c3nr33268c

Zeitz, J. O., S. Bucher, X. Zhou, L. Meile, M. Kreuzer, and C. R. Soliva. 2013. Inhibitory effects of saturated fatty acids on methane production by methanogenic Archaea. J. Anim. Feed Sci. 22:44–49.

Zhao, L., Q. Meng, L. Ren, W. Liu, X. Zhang, Y. Huo, and Z. Zhou. 2015. Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. Asian-Australasian J. Anim. Sci. 28:1433–1441. doi:10.5713/ajas.15.0091.

Zhou, X., L. Meile, M. Kreuzer, and J. O. Zeitz. 2013. The Effect of Saturated Fatty Acids on Methanogenesis and Cell Viability of Methanobrevibacter ruminantium. Archaea. 2013:1–9. doi:10.1155/2013/106916.

Zhou, Z., Z. Yu, and Q. Meng. 2012. Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. Bioresour. Technol. 103:173–179. doi:10.1016/j.biortech.2011.10.013. Available from: http://dx.doi.org/10.1016/j.biortech.2011.10.013

van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93:5856–66. doi:10.3168/jds.2010-3281. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21094759

van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94:4028–38. doi:10.3168/jds.2011-4236. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21787938

Zinn, R. A., and F. N. Owens. 1986. A Rapid Procedure for Purine Measurement and Its Use for Estimating Net Ruminal Protein Synthesis. Can. J. Anim. Sci. 66:157–166. doi:10.4141/cjas86-017. Available from:

http://pubs.aic.ca/doi/pdf/10.4141/cjas86-017

BIOGRAPHICAL SKETCH

Darren grew up in southeast Texas in a small town called Porter. From a young age he was heavily involved with agriculture, either through his family's cow-calf operations or through 4-H and FFA. After graduating from New Caney High School, Darren began his Bachelor of Science degree in College Station, TX at Texas A&M University where he received degrees in Animal Science and Agricultural Leadership and Education. Darren lived and studied for 6 months in Pirassununga, SP, Brazil. It was while working at a research feedlot in Brazil that Darren discovered his passion for research. Following graduation in December 2011, Darren travelled to Marianna, FL, the home of the North Florida Research and Education Center, where he lived and worked throughout his Master of Science and Doctor of Philosophy degrees. Darren worked under the advisement and mentorship of Dr. Nicolas DiLorenzo throughout both advanced degrees while at the University of Florida. Darren is happily married to his beautiful wife, Francine Henry. After completing his Doctor of Philosophy in December 2017, Darren joined the faculty at Texas Tech University as an Assistant Professor of Sustainable Livestock Grazing Management.