Effects of Bismuth Subsalicylate and Encapsulated Calcium-Ammonium Nitrate on
Feedlot Beef Cattle Production

by

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CHAPTER I

INTRODUCTION

With the world population growth not expected to plateau, the animal agriculture industry must adapt to the increasing demands by finding new and more sustainable production strategies. The utilization of coproducts generated from other industries has particularly become more popular, as some of these sectors grow bigger and their activities generate greater quantities of residues, such as distillers grains, from the production of ethanol (Klopfenstein et al., 2008; Popp et al., 2016). The ethanol industry is one that is expanding rapidly, and this expansion is attributed to the increasing demand for alternative sources of fuel (Klopfenstein et al., 2008). In the world, Brazil and the United States are the top ethanol producers accounting for approximately 75% of the global production. Consequently, the U.S. ethanol industry generated an estimated of 39 million metric tons of distillers grains during the 2013-2014 marketing year (Popp et al., 2016).

Ruminants have the ability to utilize distiller grains (**DG**) very efficiently (Klopfenstein et al., 2008). After being processed, the nutrients (protein, fat, carbohydrates) in corn increase their concentration approximately up to 3-fold, since the only portion of the grain that is required to produce ethanol is starch (Klopfenstein et al., 2008). The nutritional properties of DG, especially when it comes to CP concentrations, make this coproduct an excellent candidate as a feed ingredient to the cattle feeding industry, not only because of its feed characteristics, but also because of the large supply and the price relative to corn (Klopfenstein et al., 2008). However, the concentration of S is a factor that can potentially limit the inclusion level of DG in the diets of ruminants. It

has been reported that the concentration of S in DG ranges from 0.3 to 1.7%, with an average of 0.6 to 0.8% DM basis (Drewnoski et al., 2014).

Primarily, S in the body occurs in proteins that contain amino acids such as cystine, cysteine, and methionine. Among the many important roles that S has in the animal body, this element is also required to support adequate growth and metabolism of many ruminal bacteria, especially cellulolytic bacteria (Drewnoski et al., 2014). The requirements of sulfur for beef cattle are recommended to be around 0.15% to support growth (NASEM, 2016). There are challenges that have been related to high dietary levels of S in beef cattle production such as reductions in performance, absorption of trace mineral, and the incidence of the disease polioencephalomalacia (Drewnoski et al., 2014). The pathological effects connected to high levels of sulfur in ruminants are primarily caused by H_2S , a gas that is synthesized by sulfate reducing bacteria within the rumen (Gould, 1998; Drewnoski & Hansen, 2013; Drewnoski et al., 2014).

Bismuth subsalicylate has been used in humans for more than two centuries, and its development as an aid for gastrointestinal spasms turned it into Pepto-Bismol®, one of the most common remedies for upset stomach, and diarrhea (Bierer, 1990). Bismuth has been observed to bind to the S in H_2S and consequently forming bismuth sulfide B_2S_3 , therefore alleviating the negative effects of H_2S (Suarez et al., 1998). Using bismuth subsalicylate (**BSS**) as a ruminal H_2S mitigator has not been extensively evaluated until recently when Ruiz-Moreno et al. (2015) looked at the effects of different levels of BSS on in vitro ruminal fermentation and in vitro ruminal H_2S production.

Nitrate has been primarily investigated as CH_4 reducer in beef cattle production through its H sink action mechanism (Leng, 2008; Olijhoek et al., 2016; Guyader et al.,

2017). However, the combination of BSS and nitrate has been only investigated by (Henry et al., 2016 and Henry et al., 2017) where the potential of BSS and encapsulated ammonium nitrate (**eCAN**) to reduce in vitro H_2S were observed using a high forage substrate. Henry et al. (2017) also evaluated the same treatments in vivo where results showed different outcomes compared to in vitro data (Henry et al., 2016).

There is limited research with nitrate and bismuth subsalicylate as H_2S mitigator. Therefore, the following review of the literature provides an overview of the challenges that feeding high levels of dietary S engulf, as well as some alternatives that offer the possibility to overcome such challenges. Moreover, this review addresses some of the data generated with nitrate as a CH_4 mitigator, and some of the pioneering research that has been performed over the last few years with bismuth subsalicylate in ruminants and its potential to reduce ruminal H_2S production are discussed.

CHAPTER II

REVIEW OF THE LITERATURE

The importance of sulfur in the ruminant diets

Sulfur (S) is an important element found in different metabolic processes and vital components in the nutrition of the ruminant animal (McDonald et al., 2011 and Drewnoski et al., 2014). Most of the S in the animal body occurs in proteins containing the amino acids cystine, cysteine, and methionine (McDonald et al., 2011). Other essential elements that possess S in their structure are the vitamins biotin and thiamin, as well as the hormone insulin, and the vital metabolite coenzyme A (McDonald et al., 2011). Sulfur is also required to support adequate growth and metabolism of many ruminal bacteria, especially cellulolytic bacteria (Drewnoski et al., 2014).

The requirements of S in beef cattle are not well defined (NASEM, 2016); however, the NASEM (2016) recommends a 0.15% concentration in the diet (DM basis) of beef cattle to support adequate growth. It is crucial to keep in mind that the level of inclusion of S will depend on the ingredients that are part of the diet, and of course, the type of diet per se, as some sources of the sulfur element might carry it in a more available state, which could lead to toxicity issues (NASEM, 2016). Additionally, the importance of S in the structural compound chondroitin sulfate is worth mentioning due to its participation as a component in cartilage, bone, tendons, and the walls of blood vessels (McDonald et al., 2011). Sulfur-containing compounds are also crucial in elements of the respiratory process from hemoglobin all the way through to cytochromes (Drewnoski et al., 2014).

Sources of sulfur

Sarturi et al. (2013) investigated the impact of different sources of S in ruminants. Organic and inorganic sources were fed to ruminally cannulated crossbred beef steers to observe the effects of the two different sources of S on intake, intake pattern, ruminal pH, VFA profile, and ruminal hydrogen sulfide (**H₂S**) concentration. The authors discussed the critical concept of ruminally available S (**RAS**). The concept of RAS is based on the availability of S for reduction in the rumen, depending on the source of the element.

Inorganic sources have greater availability for ruminal reduction to sulfide, whereas, organic sources are less available due to their relationship with some S-containing amino acids (Sarturi et al., 2013). Once sulfate-reducing bacteria have utilized the remaining part of RAS, the S that is left can have other fates such as being part of undegradable protein fraction (Sarturi et al., 2013). Both organic and inorganic S may come from different sources. For example, sulfates present in the water, as well as ingredients included in the diet of the animals such as coproducts from the grain distillery industry. Therefore, it is recommended to monitor all sources of S that can potentially affect animal health and growth performance (Sarturi et al., 2013; Drewnoski et al., 2014)

Issues related to sulfur

There is an increasing amount of information regarding S and its effects on cattle health and performance (Drewnoski et al., 2014). The increased interest in S is indeed a consequence of the greater utilization of coproducts from the ethanol industry in the diets of ruminants (Drewnoski et al., 2014).

Gould (1998) discussed the medical disorders caused by sulfur, where the disease polioencephalomalacia (**PEM**) is thoroughly described, as well as the role of sulfur in the

incidence of this disease. Other factors that cause PEM are also defined by Gould (1998), such as thiamine deficiencies, acute lead poisoning, and water deprivation-sodium ion toxicosis. However, only sulfur-induced disorders will be discussed in this document.

Polioencephalomalacia is a neurological disorder of ruminants that is characterized by lesions of the grey matter in the brain that lead to necrosis of the cerebral cortex (Gould, 1998; Drewnoski et al., 2014). It has been demonstrated that animals that are fed greater amounts of supplemental S have a greater incidence of PEM (Gould et al., 1997). Steers fed a high S diet were diagnosed with PEM showed an elevated concentration of H_2S in their rumen fluid, which points in the direction of this compound as the leading promoter of PEM (Gould et al., 1997).

Though PEM is a well-known pathological issue that S and H_2S are related to, there are other effects that have to do with reductions in performance. It has been observed that cattle consuming a high concentrate ration are more susceptible to the harmful effects of increasing dietary S levels (Drewnoski & Hansen, 2013). An experiment was performed where cattle were switched from a corn silage based diet to a ground corn-based diet (Spears et al., 2011). Cattle were gradually transitioned from the corn silage diet to the ground corn diet, and dietary concentrations of S were 0.12%, 0.30% and 0.46% DM for the corn silage-based diet, and 0.13%, 0.31%, and 0.46% of the diet DM for the ground corn based diet (Spears et al., 2011). There was a linear decrease in DMI was observed between the low and high sulfur treatments when cattle were consuming the ground corn ration; however, no differences were observed on DMI between low and high sulfur treatment when cattle were receiving the corn silage diet (Spears et al., 2011). These changes based on diet type are likely related to

differences in ruminal pH, and the rumen microbiome populations (Spears et al., 2011 and Drewnoski et al., 2014). Moreover, beef cattle performance showed quadratic and linear effects on DMI, average daily gain (**ADG**), and gain to feed ratio (**G:F**), as the levels of S in the diet are raised (Klopfenstein et al., 2008).

Increasing levels of dietary S have been observed to have negative effects on performance and carcass quality (Richter et al., 2012). Additionally, the effects on performance such as ADG, and G:F are also related to the impact on hot carcass weight (**HCW**) where a similar response is observed (Richter et al., 2012; Klopfenstein et al., 2008). Despite the effects on HCW, longissimus muscle (**LM**) area was not affected by dietary S level. However, some effects have been observed on marbling score and fat thickness, where high S treatments appear to increase these variables (Klopfenstein et al., 2008).

Adequate mineral nutrition is critical to achieving successful cattle performance, especially in the feedlot, where cattle do not have access to other sources of this vital component of the diet. Minerals are essential for proper nerve conduction, cell signaling, energy metabolism, and play the crucial role of cofactors in enzyme activity (McDonald et al., 2011). The pathological, performance, and carcass quality effects are not the only issues related to high levels of dietary S. While most of the latter repercussions have been attributed to S becoming H₂S and affecting the health and performance of cattle, S has also been linked to mineral antagonistic mechanisms (Drewnoski et al., 2014 and Pogge et al., 2014). High dietary concentrations of S may compromise the absorption of other minerals such as Cu, Se, Zn, Ca, and Mg (Pogge et al., 2014).

Drewnoski et al. (2014) described the mechanism of action of S upon trace minerals when S interacts with molybdate and thiomolybdate is formed as a result of such interactions. The latter has a high affinity for Cu. Once Cu is bound to thiomolybdate, such compounds are unavailable for absorption, which, in consequence, will lead to a reduction in the Cu status within the body, resulting in a deficiency (Drewnoski et al., 2014; Suttle et al., 1991). Pogge et al. (2014) fed two dietary levels of S at 0.24% and 0.68% and observed the effects on daily mineral intake, fecal and urine excretion, as well as retention on beef steers. The authors reported that Cu did not change between treatments, but its fecal excretion increased leading to a reduction in retention. A similar effect was observed for Zn and Mn, which, despite observing an increase on Zn and reduction on Mn intake, the excretion and retention followed the same pattern as Cu (Pogge et al., 2014).

The apparent absorption was dramatically reduced for Cu and Zn by the greater inclusion of dietary S. However, the change in concentration of these elements within the liver at d 28 was only reduced for Zn (Pogge et al., 2014). The authors attribute the lack of effect on Cu concentration within liver to the longer life of the Cu pool, as lower plasma Cu levels were observed by d 28; however, there were no signs of depletion of liver Cu levels (Pogge et al., 2014).

Spears et al. (1991) conducted a similar experiment where decreased liver Cu concentration was observed on steers receiving the higher dietary S concentration. Plasma Cu concentrations were not affected by dietary S by day 84; however, by d 101, plasma Cu levels were lesser on the steers receiving greater dietary levels of S (Spears et al., 1991). These results were in agreement with Pogge et al. (2014), the lag observed in

plasma Cu concentration suggests that the body is absorbing Cu likely bound to thiomolybdates, which are preventing its utilization nonetheless. Additionally, it is possible that from the survival point of view, the body is recycling the Cu pool that has been stored in the liver, which resulted in lower liver levels by the end of the trials, for the animal receiving the high S treatments (Pogge et al., 2014; Spears et al., 1991; Drewnoski, 2014).

High-S diets have also been related to are the development of oxidative stress, caused by the depletion of glutathione (**GSH**; Drewnoski et al., 2014). The cellular mechanisms connected to the adverse effects of S on health are related to the reduction of the element to H₂S (Gould et al., 1997). Such mechanisms allow the input of H₂S into the bloodstream, which triggers the pathological responses, and the antagonistic effects caused by S in the rumen that influence the malfunction of enzymes such as superoxide dismutase, which are Cu and Zn dependent (Drewnoski et al., 2014).

Hydrogen sulfide

Hydrogen sulfide is a colorless gas with a characteristic rotten-egg odor that occurs naturally in petroleum, volcanic, and sulfur-spring emissions, as well as in natural gas (Dorman et al., 2002). As previously mentioned, H₂S has been implicated as one of the main drivers of the adverse effects of S in ruminants (Drewnoski et al., 2012a; Drewnoski et al., 2014; Gould, 1998). Sulfate-reducing bacteria (**SRB**) are primarily responsible for the biosynthesis of H₂S, in the rumen, sulfates can be reduced through dissimilatory or assimilatory reduction (Drewnoski et al., 2014). Dissimilatory SRB reduce sulfate through anaerobic respiration pathways to support their bioenergetic processes, and they depend on the activity of dissimilatory sulfite reductase; as a result

H₂S is formed as an end product (Drewnoski et al., 2014). Assimilatory reduction occurs when SRB reduce sulfate to H₂S and then use the latter as a substrate to produce S-containing amino acids, or cofactors such as biotin and pantothenic acid (Drewnoski et al., 2014). The population of SRB in the rumen have not been well investigated; however, they are estimated to be approximately less than one percent of the total ruminal bacterial population (Drewnoski et al., 2014).

Though Drewnoski et al. (2014) and Rees (1973) agree upon the model that proposes the direct reduction of sulfite and sulfate to H₂S through dissimilatory sulfite reductase, Kobayashi et al. (1969) have proposed a different model where other intermediary products, such as trithionate are produced and reduced to H₂S. Hydrogen sulfide is a typical product of microbial metabolism synthesized in the rumen (Gould, 1998), some involving SRB described by Sarturi et al. (2013). The sulfate pathway involves adenosine triphosphate (ATP) to form adenosine-3-phosphosulfate, which consequently reacts with ATP to produce 5'-phospho-adenosine-3'-phosphosulfate (PAPS), or it releases H₂S by dissimilatory reducing bacteria (Gould, 1998).

The assimilatory reducing bacteria utilize PAPS for the synthesis of S-containing amino acids. Populations of sulfate reducing-bacteria such as *Desulfivibrio* spp. have been observed to increase as a response to high-S diet adaptation. Moreover, these populations of bacteria have the potential of enhancing the production of H₂S in the rumen (Gould, 1998).

The exposure of ruminants to the pathological effects of H₂S is mainly through inhalation of the gases that escape from the rumen. Pulmonary damage has been observed in rats, and acute interstitial pneumonia in cattle which have been both blamed on inhaled

H₂S (Gould, 1998). Additionally, steers consuming a high sulfate diet diagnosed with diet-induced PEM had an increased concentration of H₂S in the ruminal gas cap to a much larger degree than in the rumen fluid (Gould, 1998).

The pK_a of H₂S is 7.04 (Gould, 1998). One third of H₂S exists in undissociated form at a pH of 7.4, while the remaining two thirds in the form of hydrosulfide ions (Schoonmaker and Beitz, 2012). The ruminal pH of animals consuming a high-concentrate diet is often in the range of 5.8 to 6.5 (Nagaraja & Titgemeyer, 2007), which indicates that the production of H₂S in this type of rumen environment is prone to become higher (Gould, 1998; Drewnoski et al., 2014; Drewnoski & Hansen, 2013). A decrease in ruminal pH triggers the production of H₂S (Schoonmaker and Beitz, 2012). For example, it has been reported that at pH 5.2, the percentage of H₂S vs hydrosulfide anion is 97.2 and 2.8 respectively, whereas at pH 6.8 it is 46.8 and 50.4, respectively (Gould, 1998).

Feedlot cattle appear to be more prone to S-induced PEM, which has been related to a substantial increase in ruminal concentration of H₂S, especially in the first 30 d post feedlot arrival (Drenowski & Hansen, 2013). After arrival, a drop in ruminal pH occurs, which in combination with a high level of dietary S may cause a disproportionate increase of H₂S together with a greater incidence of PEM (Drenowski & Hansen, 2013).

The PEM incidence and increase in concentration of H₂S was observed independently of prior exposure to greater levels of dietary S, perhaps delivered as a supplement during the backgrounding phase. This response may indicate that the first 3 to 4 weeks after feedlot arrival are critical, and should be closely monitored to avoid the adverse effects caused by H₂S (Drenowski & Hansen, 2013). Drenowski et al. (2012b)

reported ruminal concentrations of 12,000 mg/L or higher to be implicated in the incidence of PEM; however, concentrations above those levels have been observed without developing any symptoms of the PEM.

The exact mode of action by which H_2S acts upon the cell has not been fully elucidated; however, models of the cytotoxicity mechanisms have been proposed (Drewnoski et al., 2014; Gould, 1998; Dorman et al., 2002). Hydrogen sulfide metabolism may occur through different pathways such as oxidation, methylation, and a reaction with cytochrome c oxidase, which is a critical enzyme in the electron transport chain (ETC) within the mitochondria (Dorman et al., 2002). The latter is thoroughly described by Drewnoski et al. (2014).

Briefly, when H_2S crosses the cellular membrane, it travels to the mitochondria where it blocks cytochrome c oxidase and disrupts the ETC (Drewnoski et al., 2014). Dorman et al. (2002) observed a linear reduction in cytochrome c oxidase activity within the lungs of rats that were exposed to ≥ 30 ppm H_2S . Once the ETC is disrupted, superoxide ions ($\text{O}_2^\cdot$) are created by a combination of O_2 with leaked electrons from the ETC (Drewnoski et al., 2014). The release of $\text{O}_2^\cdot$ triggers a signal for the cell to produce Mn and Cu-Zn superoxide dismutase, which converts $\text{O}_2^\cdot$ to the less damaging compound hydrogen peroxide (H_2O_2), glutathione, Se-dependent glutathione peroxidase, catalase, or vitamins C and E turn H_2O_2 to water (Drewnoski et al., 2014).

Furthermore, H_2S can also contribute to the release of cellular Fe (Fe^{2+} and Fe^{3+}) from the protein ferritin, which can further add to reactive sulfur species, and hydroxyl radicals ($\text{OH}^\cdot$) formation via the Fenton reaction (Drewnoski et al., 2014).

Mitigation strategies for sulfur toxicity

Different approaches have been developed in effort to mitigate H₂S toxicity. Sarturi et al. (2013) described the adjusted ruminal protein S (**ARPS**) concept that considers the S that can be potentially converted into an excess of H₂S. This concept discusses the availability of S for the production of H₂S by SRB. The ARPS concept offers the opportunity to calculate how much S could potentially be degraded or bypass the rumen, which in the end can allow some level of control over these parameters (Sarturi et al., 2013; Drewnoski et al., 2014). There are differences between organic and inorganic sources of S, inorganic sources of S are assumed to be 100% available for ruminal reduction to sulfide; however, organic sources of S appear to be less available due to the attachment of S to S-containing amino acids, and its availability for reduction to sulfide will depend on the proportion of ruminal degradable protein in such ingredient. When inorganic S from ammonium sulfate and wet distiller's grains with solubles are fed, the response is the same in terms of ruminal H₂S production at 8 and 13 hours post-feeding (Sarturi et al., 2013).

Nichols et al. (2012) performed a meta-analysis where the interaction of dietary S and roughage inclusion on the incidence of PEM was evaluated. The authors reported that as dietary S concentration increased, the occurrence of PEM also increased; however, increasing the concentration of NDF in the diet reduced the number of PEM cases per day. Cattle fed a diet with 4% roughage-NDF during an S-induced PEM (**S-PEM**) challenge had an incidence of 0.56 cases per day in a 20,000 head feedlot. On the other hand, in cattle that consumed a diet with 0% roughage-NDF, PEM increased to 23 cases per day in a 20,000 head feedlot (Nichols et al., 2012).

In general, it has been estimated that the risk of S-PEM is reduced by approximately 19% for each 1% increase in the dietary roughage-NDF levels (Drewnoski et al., 2014). It is important to consider the implications upon the rumen environment by increasing the levels of dietary roughage-NDF. As previously discussed, pH influences the levels of ruminal H_2S ($\text{pK}_a=7.04$; Gould, 1998). Therefore, it is suggested that increasing roughage-NDF in the diet of cattle, the change in rumen pH is shifting more sulfide to the dissociated form (Gould, 1998; Nichols et al., 2012; Drewnoski et al., 2014).

Morine et al. (2014a) evaluated different dietary levels of NDF and the effects on ruminal concentration of H_2S in steers fed a high S diet. The authors reported a linear increase in ruminal pH, while the concentration of H_2S linearly decreased as the dietary concentrations of NDF increased from 21% to 30% (DM basis; Morine et al., 2014a). Moreover, data indicate that as inclusion of NDF in the diet is greater, the production of ruminal H_2S decreases and consequently the incidence of PEM is reduced (Morine et al., 2014a; Morine et al., 2014b; Drewnoski et al., 2014).

Thiamine has been extensively recommended as a preventive measurement for animals consuming high dietary S (Drewnoski et al., 2014). Thiamine injections have been observed to eliminate the symptoms of PEM; therefore, this treatment has become the main strategy to treat disease (Drewnoski et al., 2014). However, some cases have been observed where the lack of supplementation of the vitamin did not cause the manifestation of the of PEM, regardless of level of dietary S (Drewnoski et al., 2014). Additionally, though injections of thiamine can mitigate the expression of PEM, brain lesions have been confirmed in the original diagnosis (Drewnoski et al., 2014).

As previously discussed, the depletion of GSH in the cell has been proposed as one of the mechanisms of action that are involved when PEM is inflicted by elevated concentrations of ruminal H₂S (Drewnoski et al., 2014). The depletion of GSH forces the cell toward the utilization of other antioxidants such as vitamin C and E, as well as trace mineral-dependent enzymes such as superoxide dismutase catalases (Drewnoski et al., 2014). Therefore, the supplementation of antioxidants could also be considered a strategy to combat the adverse effects of high dietary-S (Drewnoski et al., 2014). Other strategies such as the use of ionophores, or the supplementation with Fe in the form of ferric ammonium citrate have been investigated in the efforts to control ruminal concentration of H₂S; however, the results have been variable and somewhat inconclusive (Drewnoski et al., 2014).

Bismuth subsalicylate

Bismuth subsalicylate (**BSS**) has been extensively used in humans as an aid for gastrointestinal spasms (Bierer, 1990). The first registered use of the compound was in 1733, but it was not until 1799 that bismuth was included in the literature as a potent relief for stomach pain (Bierer, 1990).

In 1900, a physician in New York manufactured a liquid formula with BSS and Zn salts for their astringency, phenyl salicylate, extract of wintergreen plant to provide some flavor, and finally a red dye so that the product could be appealing to children (Bierer, 1990). In 1918, Norwich Pharmacal distributed for the first time the remedy with the name of Bismosal, which was later changed to Pepto-Bismol (Bierer, 1990). Since its beginnings nearly two centuries ago, Pepto-Bismol now found in approximately 60% of homes in America (Bierer, 1990).

Bismuth subsalicylate is an insoluble salt that contains 58% bismuth and 42% salicylate by weight (Bierer, 1990). In the stomach, BSS dissociates and binds to other elements including S; therefore, creating bismuth and salicylate salts. Less than 0.005% of bismuth from BSS can be absorbed in the gastrointestinal tract, while the majority of it is excreted through feces; however, the salicylate portion is extensively absorbed (>90%), and discharged in the urine (Bierer, 1990; Suarez et al., 1998).

Suarez et al. (1998) evaluated the potential of BSS to reduce the concentration of H₂S in rat and human feces. The authors observed that concentrations of H₂S were linearly decreased as the dose of BSS was increased, which exposed the potential of BSS as a mitigator of H₂S within the gastrointestinal tract (Suarez et al., 1998). Additionally, BSS has been observed to have antimicrobial action over *Helicobacter pylori* and *Escherichia coli* (Suarez et al., 1998).

Bismuth subsalicylate as a mitigating strategy for hydrogen sulfide in ruminants

The use of BSS in ruminants had not been evaluated until recently when Ruiz-Moreno et al. (2015) used the compound in vitro with ruminal fluid as inoculum. Bismuth subsalicylate has been observed to reduce in vitro H₂S production to up to 99% when it was included at 4% (substrate DM; Ruiz-Moreno et al., 2015). Additionally, the addition of BSS increased the digestion of NDF and ADF; however, the carbohydrate degradation and total VFA concentration were reduced (Ruiz-Moreno et al., 2015).

Likewise, similar results were observed when BSS was used in vitro with Bahiagrass hay (*Paspalum notatum*) (Henry et al., 2016). The inclusion of bismuth at 0.33% of the substrate DM showed a 61% reduction of H₂S compared to control (0.00%

BSS), the total production of VFA was unaffected by higher inclusions of BSS (0.66% and 1.00%) while in vitro fermentation was negatively affected (Henry et al., 2016).

The data that has been generated on BSS in the area of ruminant nutrition opens the opportunity for more research with this compound. The potential offered by bismuth, as a H₂S mitigator in ruminants should be further explored, in the efforts to find more alternatives for the control of the adverse effects of the harmful gas.

Although there is data showing the effects of BSS in with rumen fluid as an inoculum (Ruiz-Moreno et al., 2015; Henry et al., 2016), the compound is still novel in the field of ruminant nutrition. To the author's knowledge, no data has been generated about the use of BSS in feedlot beef cattle performance, liver mineral concentration, nutrient digestibility, and carcass quality.

Nitrate and its use in ruminants

The use of nitrate in ruminants has been primarily investigated over the past decade as a methane (CH₄) reduction strategy (Leng, 2008; van Zijderveld et al., 2011; Guyader et al., 2017).

Nitrate has been observed to persistently reduce of up to 16% of enteric CH₄ production in grams per kilogram of dry matter intake (**DMI**) or as a percentage of gross energy intake (**GEI**; van Zijderveld et al., 2011). Gross energy losses have been implicated as one of the consequences enteric CH₄ production in ruminants (Johnson and Johnson, 1995). The concept involves the utilization of hydrogen ions (H⁺) released in the rumen for the synthesis of CH₄, these H⁺ originate during the microbial fermentation of the diet components within the rumen (Johnson and Johnson, 1995). Volatile fatty

acids are produced through several different pathways, each one destined to create a specific VFA, and depending on which VFA is being synthesized the yield of H^+ will differ (Johnson and Johnson, 1995). For example, H^+ are used as substrate for the production of propionic acid as opposed to the production of acetic acid where H^+ are released, therefore, CH_4 is affected by on the VFA profile in the rumen (Johnson and Johnson, 1995). It is estimated that if the acetic acid to propionic acid ratio (A:P) is 0.5 the loss of energy as methane would be 0%, on the other hand, if all of the carbohydrates in the rumen were converted into acetic acid, the energy loss would be nearly 33% (Johnson and Johnson, 1995). In general, however, the losses of GEI in cattle through methane are estimated to be approximately 2 to 12%, depending on different factor such as diet type, for example (Johnson and Johnson, 1995).

Janssen, (2010) thoroughly describes the chemistry, kinetics, and thermodynamics of H^+ that exist in the rumen, which is primarily influenced by microbial fermentation. The H^+ released during ruminal fermentation can occur either in the gas gap or dissolve within the rumen content where they are utilized by methanogens to produce CH_4 (Janssen, 2010).

Enteric CH_4 production in ruminants is mainly driven by the availability of H^+ and the type of diet (Johnson and Johnson, 1995; Janssen, 2010). It has been observed that the digestibility of feed plays a vital role in CH_4 production, where more digestible feeds result in reduced emissions (Janssen, 2010). The proportion of VFA is also affected by diet type, where readily degradable diets, such as beef finishing diets, are more prone to have a decreased A:P and a decreased ruminal pH (Nagaraja and Titgemeyer, 2007; Owens et al., 1998; Johnson and Johnson, 1995).

There are different fates that glucose can encounter after being metabolized in the rumen, and, depending on this, the units of CH₄ per unit of glucose metabolized will differ (Janssen, 2010). For example, if a molecule of glucose is converted to 2 acetate + 4H₂, there will 1.00 mol of CH₄ released, while the same molecule of glucose can be converted instead into 0.66 acetate + 1.33 propionate, with 0 mol of CH₄ produced (Janssen, 2010).

The mechanism of action by which nitrates reduce CH₄ production is by acting as a hydrogen sink within the rumen (Zhou et al., 2011; Olijhoek et al., 2016; Guyader et al., 2017). Theoretically, once in the rumen, nitrate is reduced to nitrite ($\text{NO}_3^- + \text{H}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$), and then it is further reduced to ammonium ($\text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$; Olijhoek et al., 2016). This reaction is precisely what attributes the hydrogen sink properties to nitrate and that consequently translate into the reduction of CH₄ where 1 mol of nitrate is capable of reducing 1 mol of CH₄ (Leng, 2008). Additionally, the reduction of nitrate to nitrite appears to be more thermodynamically favorable than the reduction of CO₂ to CH₄ (Olijhoek et al., 2016; van Zijderveld et al., 2010).

When nitrate reduction and carbon dioxide reduction are compared, the reduction of nitrate to nitrite ($\Delta G_0 = -130$ kJ) together with the further reduction of nitrate to ammonium ($\Delta G_0 = -371$ kJ) yield more energy than the reduction of carbon dioxide to CH₄ ($\Delta G_0 = -16.9$ kJ; Olijhoek et al., 2016; van Zijderveld et al., 2010).

This reduction mechanism could represent a route of hydrogen disposal when nitrate is available in the ruminal environment in adequate quantities. Ammonia can also be a byproduct of nitrate reduction in the rumen that microbes can utilize for the synthesis of microbial crude protein (**MCP**), which offers the possibility of implementing

the addition of nitrate as non-protein nitrogen (**NPN**) in the diets of cattle (Olijhoek et al., 2016; van Zijderveld et al., 2010).

Guyader et al. (2017) described the concept of redirecting metabolic hydrogen (**[2H]**) by inhibiting methanogenesis. The release of [2H] occurs in the rumen when the ruminal microorganisms ferment the feed consumed by the animal. The [2H] is transferred to different acceptors to ensure the continuity of fermentation by re-oxidizing reduced co-factors (Guyader et al., 2017). There are several processes that depend on the existence of [2H] within the rumen environment, such as MCP, the synthesis of some VFA (propionate for example), and the synthesis of CH₄ (Guyader et al., 2017; Janssen, 2010; Olijhoek et al., 2016). Ultimately, the flow of [2H] is imperative to the energy metabolism driving ruminal fermentation, which has a major impact on ruminant nutrition (Guyader et al. 2017).

The importance of [2H] highlights the impact that enteric CH₄ production may have on cattle performance. As previously discussed, 2-12 % gross energy can be lost in ruminants through CH₄ emissions (Johnson and Johnson, 1995). Therefore, the strategies to reduce these impacts offer the possibility of overcoming the effects on performance while mitigating the impact of animal agriculture on the planet.

Guyader et al. (2017) proceeded to evaluate the effects of nitrate, nitrooxypropanol, and anthraquinone on [2H] fate when CH₄ is reduced, using the Rumen Simulation Technique (RUSITEC) with treatments being 1.07 g of calcium ammonium nitrate/vessel/d, 5 mg of nitrooxypropanol/vessel/d, and 12.3 mg of 9, 10-antraquinone/vessel/d; all treatments were made isonitrogenous with urea. The results from this research show the reduction of CH₄ by nitrate and nitrooxypropanol for as much

as 75%, and anthraquinone with a 26% reduction (Guyader et al., 2017). The decrease of CH₄ by nitrate resulted in a higher efficiency of the microbes to produce MCP (1.16 fold compared to control), which supports the theory explained by Olijhoek et al. (2016) and van Zijderveld et al. (2010) that the reduction of nitrate offers a source of CP for the ruminal microbes.

While the hydrogen sink mechanisms have been discussed as being the most influential aspect on the reduction of CH₄, nitrate has also been implicated in the modification of microbial populations in the rumen (Guyader et al., 2017; Zhou et al., 2011; Duin et al., 2016). Zhou et al. (2011) observed a 97% reduction in vitro ruminal methanogen population when concentrations as low as 12 µmol/ml were added to the media. These findings indicate the powerful antimicrobial effect that nitrate may have over methanogens; however, other findings attribute this to nitrite, instead of nitrate (Duin et al., 2016).

In the rumen *Archaea* are the main contributors to CH₄ production (Zhou et al., 2011; Duin et al., 2016). These microorganisms capture dissolved H⁺, and with the help of the key enzyme methyl-coenzyme M reductase (**MCR**), synthesize the molecule of CH₄. It has been observed that 3-nitrooxypropanol inhibits MCR; however this effect occurs when 3-nitrooxypropanol is converted to nitrite and nitrate, where nitrite has been observed to be the responsible for the inhibition of MRC, therefore, elucidating the antimicrobial effect that this molecule can have upon methanogens (Duin et al., 2016). Attention must be paid when a compound with antimicrobial properties such as 3-nitrooxypropanol is fed to ruminants, as it can affect other microorganisms that are necessary for the proper digestion of feed (Duin et al., 2016). Therefore, the effects of 3-

nitrooxypropanol on nonmethanogenic bacteria in the rumen have been assessed without observing adverse effects (Duin et al., 2016).

Newbold et al. (2014) conducted an experiment to evaluate the effects of dietary nitrate on methane emission in Holstein steers and performance in Nellore bulls were evaluated. The authors reported a linear reduction of CH₄ production as the inclusion of nitrate increased from 0 to 3% of the diet DM (Newbold et al., 2014). Furthermore, linear decrease in feed intake as the levels of dietary nitrate were increased without affecting ADG of Nellore bulls, this resulted in a linear increase in G:F as dietary nitrate levels increased (Newbold et al., 2014).

Despite the potential of nitrate as a mitigator of enteric CH₄ while improving cattle performance (Newbold et al., 2014), there is a concern expressed regarding the toxicity related to nitrite accumulation in the rumen. The use of nitrate in ruminant diets may lead to the occurrence of methemoglobinemia. This condition is caused by the oxidation of the ferric iron in hemoglobin, which blocks the ability of the molecule to carry oxygen (van Zijderveld et al., 2011). The oxidation of the hemoglobin molecule is mainly caused by nitrite, an intermediary of the reduction of nitrate when nitrite enters the bloodstream (van Zijderveld et al., 2011).

It is recommended to adapt animals that will be receiving nitrate as the primary source of NPN in the diet (Newbold et al., 2014). A gradual introduction to this type of additive allows the rumen microorganisms to adapt and increase their capability to convert nitrite to ammonia (van Zijderveld et al., 2011).

Several adaptation strategies to nitrate-based diets have been implemented (Olijhoek et al., 2016; van Zijderveld et al., 2011; Newbold et al., 2014). Some of them vary in length and steps of adaptation, going from a week to a month with gradual increases of the nitrate concentration (Olijhoek et al., 2016; van Zijderveld et al., 2011; Newbold et al., 2014). Regardless of the adaptation strategy, it is necessary for ruminants to go through this step to avoid the toxic effect that may occur with the use of nitrate in the diet.

Combination of Nitrate with BSS

Several types of nitrate have been investigated over the past years, whether it is calcium ammonium nitrate (Olijhoek et al., 2016; Henry et al., 2016) or encapsulated calcium ammonium nitrate (Lee et al., 2017; Henry et al., 2017). The results indicate that nitrate is a viable option as NPN source for ruminants, which is shown with improvements in performance and the reduction of enteric CH₄ production; however, there is little research where nitrate has been considered as a tool to reduce the negative effects of H₂S in ruminants. Henry et al. (2016) evaluated the combination of BSS and encapsulated calcium ammonium nitrate (**eCAN**), there was a 61% reduction of in vitro H₂S when BSS was added at 0.33 % compared to 0.00% of the substrate DM in combination with 2.4% of the substrate DM. However, Henry et al. (2017) observed differing results in vivo where concentrations of H₂S in the rumen gas cap were not affected by treatment. The lack of data about nitrate and BSS, especially as a H₂S mitigators in ruminants, opens the possibility for further investigation with these type of compounds in the field of ruminant nutrition.

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CHAPTER III

THE EFFECTS OF BISMUTH SUBSALICYLATE AND CALCIUM-AMMONIUM NITRATE ON IN VITRO FERMENTATION OF A HIGH CONCENTRATE DIET SUBSTRATE

ABSTRACT

An experiment was performed to determine the effects of bismuth subsalicylate (BSS) and calcium-ammonium nitrate (CAN) on in vitro fermentation of a high concentrate (87% concentrate, DM basis) substrate. Serum bottles (125-mL) containing 20 mL of a 2:1 buffer:ruminal fluid inoculum and 0.2 g of substrate were incubated for 24 h at 39 °C under constant agitation (60 rpm). Four ruminally cannulated steers were used as donors and each donor was considered as block. Treatments were arranged in a 2 × 2 factorial with the factors being BSS (0 or 0.33% substrate dry matter [DM]) and CAN (0 or 2.22% substrate DM). All treatments were made isonitrogenous with urea. In vitro organic matter digestibility (IVOMD) was determined simultaneously in separate 100-mL centrifuge tubes, the procedure consisted of 24 h of ruminal fermentation at 39 °C under constant agitation (60 rpm) followed by 48 h of post-ruminal digestion where 5 mL of a 20% HCl and 2mL of a 5% pepsin solution were added . Data were analyzed using the MIXED procedure of SAS with the fixed effect of BSS, CAN, BSS × CAN, and the random effect of donor. There were no differences observed for in vitro organic matter digestibility ($P > 0.230$). Ammonia-N tended to increase ($P = 0.092$) when CAN was used as N source rather than urea. An interaction ($P < 0.01$) was observed for total gas production (TGP). When CAN was included, without BSS, TGP was increased ($P < 0.01$); however, the combination of CAN with BSS did not affect TGP ($P = 0.845$) when compared to the combination of urea and BSS. There was a tendency for an interaction

regarding the production of H₂S ($P = 0.055$); however, there were no treatment mean differences ($P > 0.283$). The inclusion of BSS and CAN did not affect ($P > 0.624$) final pH of in vitro ruminal fermentation. There was a tendency for non-protein nitrogen source (**NPNS**) to affect NH₃-N levels where the addition of CAN tended to increase NH₃-N by 13%. No effects were observed on total VFA production ($P > 0.10$). Acetate molar proportion tended to increase by 4% when CAN was added as the NPNS which consequently affected A:P where treatments with CAN showed greater A:P ($P < 0.01$). The combination of CAN and BSS did not negatively affect fermentation parameters such as IVOMD and TGP; however, a reduction in H₂S with the combination of BSS and CAN indicate potential benefits for feedlot cattle.

Keywords: Nitrate, bismuth subsalicylate, total gas production, organic matter digestibility.

INTRODUCTION

Enteric methane (**CH₄**) generated in the gastrointestinal tract of ruminants is one of the greatest greenhouse gas (**GHG**) sources that exist in the livestock industry (Caro et al., 2016). Additionally, it has been discussed that CH₄ may affect performance by reducing the efficiency of energy utilization by the animal, as it can account for 2-12% of gross energy intake (**GEI**) lost in ruminants (Johnson and Johnson, 1995). Nitrate has been investigated in ruminants as a potential CH₄ mitigator over the past decade (Newbold., 2014; Olijhoek et al., 2016; Guyader et al., 2017). The H⁺ sink mechanism that nitrate inflicts upon the ruminal environment has driven researchers to test this compound at different dietary levels, which has been observed to effectively reduce enteric CH₄.

Sulfur (**S**) is an important elements included in the nutrition of ruminants. The relevance of S in the body and in several metabolic processes, including the adequate growth of some ruminal bacteria, position this element as an essential part of the diet in cattle production (McDonald et al., 2011, Drewnoski et al., 2014). However, when S is provided above dietary requirements, some adverse effects on performance and health may start to appear in the animal. When S is converted to hydrogen sulfide (**H₂S**), health issues, such as the disease polioencephalomalacia (**PEM**) have been observed (Gould, 1998; Drewnoski et al., 2014). Bismuth subsalicylate (**BSS**) has been used in humans for more than a century to control gastrointestinal spasms (Bierer, 1990). The potential of BSS to reduce H₂S has been investigated in humans and rats, which has proven to be very effective (Suarez et al., 1998); however, only a few laboratories have investigated the role

of BSS as H₂S mitigator in ruminal environments (Ruiz-Moreno et al., 2015; Henry et al., 2016).

The objective of this experiment was to determine the effect of calcium ammonium nitrate (**CAN**) and BSS on in vitro fermentation, CH₄ and H₂S production using a high concentrate substrate (87% concentrate, DM basis).

MATERIALS AND METHODS

All experimental procedures involving live animals during this study were conducted at the Texas Tech University New Deal Research and Education Center located in Idalou, TX. Animals were handled in compliance with the regulations of the International Animal Care and Use Committee (**IACUC**).

Treatments, Substrate, and Experimental Design

A randomized complete block design with a 2 × 2 factorial arrangement of treatments was used. Treatments were as follows: CTL (urea at 0.74% of substrate DM); NIT (CAN at 2.22% of substrate DM); UBS (urea at 0.74% and BSS at 0.33% of substrate DM); NITBS (CAN at 2.2% and BSS at 0.33% of the substrate DM). All treatments were formulated to be isonitrogenous by adding urea.

The substrate utilized was formulated as a commercial high-concentrate diet with 87% concentrate on a DM basis (Table 1.1). Prior to mixing of the substrate, all ingredients were dried separately in air forced dryer at 55°C for 24 h. Urea and CAN were dissolved in reversed osmosis filtered water and were respectively added to the substrate at their predetermined inclusion levels at the moment of incubation. On the other hand, BSS was incorporated into the substrate during mixing.

In Vitro Incubations

In vitro organic matter digestibility and batch culture procedures were performed simultaneously using the same substrate, treatments, and inoculum. Four ruminally cannulated steers were used as donors. Rumen fluid was extracted from each donor and kept in separate containers. After collection, the containers with rumen fluid were promptly transported to the Sustainable Ruminant Nutrition Laboratory located at Texas Tech Animal and Food Sciences Building, where they were separately mixed in a 2:1 McDougall's buffer to ruminal fluid mixture to be used as inoculum (McDougall, 1948). For the IVOMD, a modified version of the procedure developed by Tilley and Terry (1963) was used where 0.2 g of substrate and 20 mL of a 2:1 buffer to ruminal fluid inoculum were incubated in 100 mL plastic centrifuge tubes at 39 °C under constant agitation (60 rpm) for 24 h. Two blank tubes were also incubated without added substrate for IVOMD calculations, and all tubes were capped with a rubber stopper equipped with a 16-G needle that allowed the gases to escape. Post-ruminal digestion was simulated by the addition of 6 mL of 20% HCl and 2 mL of a 5% pepsin solution were added before the tubes were placed back in the incubator for a subsequent 48 h period. Once incubation was completed, the tubes were extracted from the incubator and contents were filtered through previously labeled and weighed P8 filters (Fisher Scientific, Pittsburgh, PA). Filters were then folded and placed in a previously weighed crucible before being dried at 100 °C for 24 h. Finally, filters were combusted in a muffle furnace at 600°C for 6 h before placing them back at 100°C for at least 6 h to record hot weights to calculate IVOMD.

The batch culture consisted of a similar procedure as described for IVOMD with the difference that batch culture bottles did not undergo a post-ruminal digestion step. Here, 0.2 g of substrate plus 20 mL of a 2:1 buffer to ruminal fluid were added into a 125-mL serum bottle. The bottles were capped and incubated for 24 h at 39 °C under constant agitation (60 rpm). Once 24 h passed, the bottles were taken out of the incubator and placed into ice for 15 min to stop microbial fermentation. After microbial fermentation was finished, bottles were allowed to reach room temperature. A 10-mL gas sample for CH₄ and another 5-mL for H₂S production were taken before the bottle content was extracted (inoculum plus substrate and treatment) but after the gas cap was exhausted for total gas production (**TGP**) measurements. Final pH of the inoculum was measure, and after adding 0.2 mL of H₂SO₂ into the bottle content, two aliquots were stored in 12 × 75 mm polypropylene disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) for, NH₃-N, and volatile fatty acids (**VFA**) analyses.

Total Gas Production, CH₄ Production, H₂S Production, and NH₃-N Analyses

Total gas production (**TGP**) was measured from each serum bottle with an inverted burette filled with water. The gas was released from each bottle by connecting them to the burette with a needle in order to measure TGP. The amount of water displaced by the gas was recorded. To measure CH₄ concentration, gas samples were analyzed with a gas chromatograph (Trace 1310 Gas Chromatograph, Thermo Scientific, Waltham, MA) using flame ionization and a capillary column. Temperatures of the injector, column, and detector were 80, 60, 250 °C respectively.

For H₂S concentration analysis, 5 mL of gas sample were injected into 15-mL evacuated tubes (BD Vacutainer, Franklin Lakes, NJ) that contained 5 mL of alkaline water as described by Smith et al. (2010). The tubes were vigorously shaken to allow proper dispersion of the gas in the alkaline water. A 0.5-mL injection of *N, N* dimethyl-*p*-phenylenediamine sulfate was inserted into the tubes followed by 0.5 mL of a ferric chloride solution. Tubes were shaken vigorously once again, and were allowed 30 min for the reaction to occur. Absorbance was read in a 96-well, flat-bottom plate at 665 nm using a plate reader (SynergyHT Microplate Reader, BioTek Instruments Inc., Winooski, VT).

To measure NH₃-N concentration, the phenol-hypochlorite assay was performed as described by Broderick and Kang (1980). Samples were centrifuged at 10,000 × *g* for 15 min at 4 °C. Phenol reagent (1mL) was pipetted into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). A 20 µL aliquot of the supernatant was transferred into the phenol containing tubes. Tubes were then vortexed, and 0.8 mL of a hypochlorite solution was added to the mixture before the tubes were vortexed once again. All culture tubes were then covered with glass marbles and placed in a water bath at 95 °C for 5 min. After boiling, contrarily to the original protocol, 200 µL were transferred into a 96-well flat-bottom plate, and absorbance was measured at 665 nm using a plate reader (SynergyHT Microplate Reader, BioTek Instruments Inc., Winooski, VT).

Volatile Fatty Acid Analysis

The concentration of VFA was determined in a water-based solution using an ethyl acetate extraction. Samples were centrifuged at 10,000 × *g* for 15 min at 4 °C. After

centrifugation, 2 mL of the supernatant were mixed with 0.4 mL of a metaphosphoric:crotonic acid (5:1) solution (crotonic acid was the internal standard) and samples were frozen overnight. The next day, samples were thawed and centrifuged again at $10,000 \times g$ for 15 min at 4 °C. The supernatant was extracted and transferred into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) where ethyl acetate was added to form a 2:1 ethyl acetate:supernatant mixture. Tubes were then shaken vigorously for proper mixing followed by a 5 min rest to allow the separation of ethyl acetate. Lastly, a subsample from each culture tube was extracted and transferred into 2 mL vials (screw vial, Thermo Scientific, Waltham, MA) before analysis. A gas chromatograph (Trace 1310 Gas Chromatograph, Thermo Scientific, Waltham, MA) equipped with a flame ionization detector and a capillary column (CP Wax 58 25m × 0.32 mm, part #CP7767, Agilent Technologies, Santa Clara, CA) was used to measure VFA concentration. The column temperature was maintained at 110 °C, and the injector detector temperatures were 200 and 350 °C, respectively.

Statistical Analysis

This experiment was analyzed as a randomized complete block design with a 2 × 2 factorial arrangement of treatments with the factors being BSS (0 or 0.33% substrate DM) and CAN (0 or 2.22% substrate DM). Data were analyzed using the MIXED procedure of SAS (SAS version 9.4; SAS Inst., Cary, NC) with the fixed effects of BSS, non-protein nitrogen source (**NPNS**), and the interaction of BSS × NPNS. The donor was considered as block and block was included as the random effect. Tukey-Kramer adjustment was used for multiple comparisons; significance was declared at $P \leq 0.05$ and tendencies were discussed at $0.05 > P \geq 0.10$.

RESULTS AND DISCUSSION

In Vitro Organic Matter Digestibility and Final pH

There were no effects ($P > 0.230$) of treatment observed on IVOMD, nor final pH ($P \geq 0.754$; Table 1.2). The effects of nitrate on in vitro fermentation parameters such as IVOMD have been observed previously by Henry et al. (2016) where the inclusion of calcium-ammonium nitrate at levels of 2.4% (DM basis) have shown linear reductions on IVOMD when compared to urea. The addition of BSS at 0.33%, 0.5%, and 1% has been observed to have no impact on pH in the past (Henry et al., 2016). However, IVOMD has been negatively affected when BSS is added above 0.33% of the substrate DM (Ruiz-Moreno et al., 2015; Henry et al., 2016). Moreover, data from Henry et al. (2016) indicate that when BSS is added in vitro at 0.33% of the substrate DM, IVOMD is not affected compared to control, which is in agreement with the current experiment. The lack of effect on final pH ($P \geq 0.754$) can be related to the lack of effect on IVOMD ($P > 0.230$), normally when IVOMD is affected, pH can be a direct indicator of such event, which can be observed in the data from the current experiment where both variables did not change. Furthermore, data from this experiment indicates that BSS at 0.33% and CAN at 2.2% of the substrate DM can be added without affecting IVOMD, which opens the opportunity for more research with these compounds.

Total gas, CH₄ and H₂S Production

A BSS $\times$ NPNS interaction ($P < 0.01$) was observed for TGP. When BSS was combined with urea, TGP was increased compared to CTL (Table 1.2). No differences were observed for TGP between NIT and NITB ($P > 0.10$). No main effects ($P > 0.159$) nor interactions ($P = 0.974$) were observed for CH₄ production (Table 1.2). These results

were unexpected as other data from our laboratory and scientific literature has indicated reductions in in vitro CH₄ production, and in vivo CH₄ production when nitrate is used (Olijhoek et al., 2016; Guyader et al., 2017; Henry et al., 2016). Additionally, Henry et al. (2016) observed linear reductions of in vitro CH₄ production when both CAN and BSS were added, which is in disagreement with the results observed in this experiment. Moreover, nitrate has been discussed to act as H⁺ sink in ruminal environments (Olijhoek et al., 2016). Through this mechanism, nitrate reduces methane production. Zhou et al. (2012) observed a significant reduction in in vitro gas and CH₄ production when nitrate was added. Likewise, Guyader et al. (2015) reported reductions in CH₄ production using a RUSITEC system, and a greater mitigation effect of CH₄ by nitrate when it was compared to 3-nitrooxypropanol, and anthraquinone.

There was a tendency for an interaction ($P = 0.055$) between BSS and NPNS on H₂S production where the addition of BSS with urea tended to increase H₂S production when compared to CTL; however, the opposite occurred when BSS was combined with nitrate where NITB tended to produce less H₂S than NIT (Table 1.2). These results are not in accordance with previous data that has indicated linear reductions on in vitro H₂S production, independent of NPNS, when BSS is added at concentrations of 0.33% of the substrate DM or greater (Ruiz-Moreno et al., 2015; Henry et al., 2016). Suarez et al. (1998) also observed a significant reduction of H₂S in human stool and rat cecum when BSS was administered to the subjects. Bismuth was utilized to mitigate H₂S by binding to S; therefore, a reduction in H₂S by BSS should be expected. However, the results obtained in this experiment do not show the same response observed by other researchers (Henry et al., 2016; Ruiz-Moreno., 2015). The NASEM (2016) recommends at least

0.15% levels of dietary S in the diet of beef cattle to support adequate growth. Ruiz-Moreno et al. (2015) utilized a substrate with a 0.44% of dietary S, whereas the substrate used in the current experiment contained 0.21% S. Although dietary S in the substrate used exceeds the recommendations of the NRC, perhaps a greater concentration or availability (0.14 ruminally available S used) of S was necessary in order to observe the effects expected from BSS on H₂S.

VFA Production and NH₃-N Concentration

Volatile fatty acid results are presented on Table 1.3. No effects were observed for total VFA production ($P > 0.05$). Previous reports have indicated similar response when CAN was provided up to 2.4% (Henry et al., 2016); however, when BSS has been added up to 1% total VFA production has been linearly decreased (DM basis; Henry et al., 2016). There was an effect of NPNS ($P = 0.05$) on acetate molar proportion where CAN increased proportion of acetate by 2 percentage units when compared to CTL and UBS. Similar results have been observed where data show an increase in acetate molar proportion with reductions in propionate molar proportion by nitrate (Zhou et al., 2012).

The lack of an effect on VFA molar proportions has also been observed where CAN has been added at 2.4% DM basis (Henry et al., 2016). Furthermore, these authors reported that as levels of BSS increase from 0.00 to 0.33% of the substrate DM, the proportions of acetate and propionate are quadratically affected (Henry et al., 2016). Similarly, Ruiz-Moreno et al. (2015) observed that molar proportions of acetate was increased when BSS was added at 4% (DM basis), followed by a reduction on molar proportion of propionate, when BSS was added at 4% of the substrate DM.

Acetate to propionate ratio was affected by NPNS ($P < 0.01$) where treatments with CAN showed a greater A:P compared to the treatments with urea. Zhou et al. (2012) also observed that increasing levels of nitrate increase A:P. Additionally, Guyader et al. (2017) observed a greater A:P when nitrate was compared to other CH₄ mitigators. However, Henry et al. (2016) reported that the addition of CAN did not affect A:P, but BSS quadratically affected this variable. The effect of nitrate on A:P observed in previous data can be attributed to utilization of H₂ in the reduction of nitrate to ammonia (Veneman et al., 2015). Propionate production requires H₂ as a substrate, therefore, it has been suggested that the decrease in H₂ caused by nitrate could potentially reduce propionate production (Veneman et al., 2015). Moreover, when rumen microbial populations from cattle consuming nitrate have been evaluated, it has been observed that propionate producing bacteria are reduced; however, this effect could be due to the antimicrobial effects of nitrate, or by the capture of H₂ when nitrate is reduced (Veneman et al., 2015).

There was a tendency for NPNS ($P = 0.092$) to affect concentration of NH₃-N where the addition of CAN tended to increase the concentration of NH₃-N by 13% (Table 1.2). This response is expected as ammonia can be synthesized as an intermediary in the reduction of nitrate to ammonium ($\text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+ \rightarrow \text{NH}_3^+ \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$) (Olijhoek et al., 2016). Zhou et al. (2012) also observed an increase in NH₃-N concentrations when nitrate was added; however, Henry et al. (2016) reported a different outcome where CAN linearly decreased NH₃-N concentrations. Ruiz-Moreno et al. (2015) observed an 8% reduction in NH₃-N when BSS was added at 0.5% or higher, which indicates that concentrations of BSS greater than 0.33% DM basis may affect NH₃

production. The data observed in the current experiment indicates that the addition of BSS at 0.33% DM basis does not affect $\text{NH}_3\text{-N}$ concentration ($P = 0.551$), which is in agreement with data observed previously by Henry et al. (2016).

The data observed in the current experiment show an increase in A:P ($P < 0.01$) as well as a tendency for greater levels of $\text{NH}_3\text{-N}$ ($P = 0.092$) when CAN was the NPNS. Although H_2 concentration was not measured, perhaps the shift on A:P generated more H^+ that were then utilized for the synthesis of NH_3^+ by the nitrate-reducing microbial population. Janssen (2010) discussed the differences in H^+ production depending on the VFA profile within the ruminal environment. When more H^+ is synthesized, ruminal microorganisms begin to act upon this ions by directing them to H^+ utilizing pathways such as CH_4 . In this case, nitrate was added as a CH_4 mitigation treatment to act as a H^+ sink. Regarding the effects on VFA, CH_4 , and TGP, the authors have not found an explanation as of why this outcome occurred, as previous research has shown different results.

CONCLUSIONS

The results observed in this experiment show that CAN and BSS can be added to the diet of ruminants at levels of 2.2% and 0.33% of the diet DM for CAN and BSS, respectively, without negatively affecting ruminal fermentation. Some of the results obtained from this study do not match previous studies performed with the same compounds. There are very few reports that have evaluated the effects of CAN in combination with BSS on in vitro ruminal fermentation, which opens the opportunity for more research with these compounds to elucidate some of the effects observed in this trial.

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TABLES**Table 1. 1.** Description of substrate and treatments for experiment 1 (DM basis)

Item ²	Treatments ¹			
	CTL	NIT	UBS	NITBS
Ingredients, % DM				
Steam-flaked Corn	63.09	63.09	62.74	62.74
Wet corn gluten feed	28.45	28.45	28.44	28.44
Cottonseed, Hulls	7.16	7.16	7.16	7.16
Limestone	1.30	1.30	1.30	1.30
Urea	0.74	—	0.74	—
CAN	—	2.20	—	2.20
BSS	—	—	0.33	0.33
Nutrient composition, % DM ³				
DM	88.70			
CP	13.52			
OM	96.20			
NDF	17.70			
ADF	7.20			
TDN	81.00			
NE _m , Mcal/kg	2.01			
NE _g , Mcal/kg	1.36			
Ca	0.66			
P	0.37			
Mg	0.23			
K	0.76			
S	0.21			

¹CTL = Control (Urea at 0.74%); NIT = Nitrate (CAN at 2.2%); UBS = Urea-Bismuth (Urea at 0.74% and BSS at 0.33%); NITBS = Nitrate-Bismuth (CAN at 2.0% and BSS at 0.33%)

²CAN = Calcium-ammonium nitrate; BSS = Bismuth subsalicylate

³Nutrient composition analysis was only performed on the basal substrate.

Table 1. 2. Effect of calcium-ammonium nitrate and bismuth subsalicylate on in vitro fermentation parameters

Item	Treatments ¹					<i>P</i> -value ²		
	CTL	NIT	UBS	NITBS	SEM ³	NPNS	BSS	BSS × NPNS
TGP ⁴ , mL/g inc. OM	159.33	220.01	223.31	221.28	32.632	0.029	0.016	0.005
IVOMD ⁵ , %	70.40	72.50	66.09	70.50	5.197	0.230	0.241	0.661
pH	5.95	5.95	5.95	5.97	0.118	0.754	0.624	0.788
CH ₄ , mmol/g inc. OM	0.13	0.19	0.18	0.20	0.082	0.477	0.644	0.753
NH ₃ -N, mM	5.31	5.86	5.39	6.25	0.968	0.092	0.551	0.687
H ₂ S, μmol/g ferm. OM	3.76	6.47	5.67	3.99	1.085	0.617	0.781	0.055

¹CTL= Control (Urea at 0.74%); NIT= Nitrate (CAN at 2.2%); UBS= Urea-Bismuth (Urea at 0.74% and BSS at 0.33%); NITBS= Nitrate-Bismuth (CAN at 2.0% and BSS at 0.33%).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between BSS and NPNS.

³ $n = 4$ experimental units/treatment mean.

⁴TGP= Total gas production as mL/g of OM after 24h.

⁵IVOMD= In vitro organic matter digestibility.

Table 1. 3. Effect of calcium-ammonium nitrate and bismuth subsalicylate on in volatile fatty acid proportions

Item	Treatments ¹					<i>P</i> -value ²		
	CTL	NIT	UBS	NITBS	SEM ³	NPNS	BSS	BSS × NPNS
VFA, mol/100 mol								
Acetate	50.50	53.47	52.12	53.26	3.278	0.052	0.464	0.346
Propionate	29.48	30.03	30.98	30.29	5.330	0.908	0.179	0.326
Butyrate	13.88	10.48	10.88	10.44	2.572	0.215	0.317	0.331
BCVFA ⁴	2.83	3.20	3.17	3.19	0.708	0.318	0.401	0.372
Valerate	2.37	2.44	2.47	2.42	0.904	0.762	0.292	0.178
Total VFA	115.69	125.87	120.22	125.45	8.312	0.293	0.773	0.728
A:P ⁵	1.91 ^a	2.02 ^b	1.89 ^a	1.99 ^b	0.451	0.003	0.456	0.758

¹CTL= Control (Urea at 0.74%); NIT= Nitrate (CAN at 2.2%); UBS= Urea-Bismuth (Urea at 0.74% and BSS at 0.33%); NITBS= Nitrate-Bismuth (CAN at 2.0% and BSS at 0.33%).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$.

³ $n = 4$ experimental units/treatment mean.

⁴BCVFA= Branched chain volatile fatty acids: isobutyrate + isovalerate + 2 methylbutyrate.

⁵A:P= acetate to propionate ratio.

^{a,b}Means with different letters are significant

CHAPTER IV

THE EFFECTS OF BISMUTH SUBSALICYLATE AND ENCAPSULATED CALCIUM-AMMONIUM NITRATE ON BEEF CATTLE PERFORMANCE, NUTRIENT DIGESTIBILITY, LIVER MINERAL CONCENTRATION, AND CARCASS CHARACTERISTICS

ABSTRACT

Two hundred Angus steers (Body weight [BW]= 385 ± 27 kg) were used to determine the effects of bismuth subsalicylate (**BSS**) and encapsulated calcium-ammonium nitrate (**eCAN**) on feedlot beef cattle performance, apparent total-tract nutrient digestibility, liver mineral concentration, and carcass characteristics. Steers were blocked by BW and allocated in 50 pens (4 steers/pen) where treatments were randomly assigned to each pen within block. Treatments were as follows: **CTL** (Urea at 0.70% with 0.28% ruminally available sulfur [**RAS**]), **NIT** (eCAN at 2.0% with 0.28% RAS), **UBS** (Urea at 0.70% and BSS at 0.33% with 0.28% RAS), **NITBS** (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS), and **LOW-S** (Urea at 0.70% with 0.14% RAS; Table 2.1). Steers were fed for 171 d and were weighed every 35 d. Dry matter intake (**DMI**) was recorded weekly. Feed and fecal samples were collected from d 44 to 51 to determine apparent total-tract digestibility of nutrients and liver tissue samples were taken on d -5 and 124 from one randomly selected animal per pen. Carcass data was collected at a commercial abattoir by trained personnel. The MIXED and GLIMMIX procedure of SAS were utilized to analyze continuous and categorical data respectively, and contrast were used for a better interpretation of the data. Significance was declared $P \leq 0.05$ and tendencies were discussed at $0.05 < P \leq 0.10$. Cattle consuming urea as non-protein nitrogen source (**NPNS**) had greater final body weight (**FBW**; $P = 0.045$) than cattle

consuming nitrate. After 171 d on feed, there was a NPNS effect where eCAN reduced DMI ($P < 0.001$) and average daily gain (**ADG**; $P = 0.041$) but did not affect feed efficiency (**G:F** $P = 0.876$). Bismuth subsalicylate tended to reduce DMI ($P = 0.095$) but did not affect ADG ($P = 0.436$) or G:F ($P = 0.876$). The effect of S was also observed ($P = 0.042$) where LOW-S cattle consumed less feed than CTL. There was a tendency ($P = 0.056$) for BSS effect to increase digestibility of DM. Additionally, a BSS effect ($P = 0.045$) was observed on OM digestibility (**OMD**) where treatments with BSS had greater OMD than non-BSS treatments. There was a tendency for an interaction ($P = 0.055$) on CP digestibility (**CPD**) where UBS tended to have greater CPD compared to CTL. A tendency for an interaction ($P = 0.083$) was also observed for ADF digestibility (**ADFD**), NIT tended to have greater ADFD than NITBS; however, CTL tended to have lesser ADFD than UBS. Cattle consuming urea had greater hot carcass weight ($P = 0.032$), and calculated yield grade was impacted by a BSS $\times$ NPNS interaction ($P = 0.019$). A reduction of final liver Cu concentration was observed ($P = 0.042$) where BSS reduced the concentration of Cu by 58%. Finally, a tendency ($P = 0.073$) for a BSS effect was observed on Cu concentration change, most likely due to the effect observed on final concentration of Cu ($P = 0.042$). Even though the final levels of Cu in the liver were within the requirements of beef cattle, it is recommended to be cautious and add a source of Cu in the feed to avoid any deficiencies. Based on the data observed in this experiment, more research should be performed with nitrate and BSS in order to elucidate some of the effects observed in the feedlot.

Keywords: encapsulated calcium-ammonium nitrate, bismuth subsalicylate, performance, digestibility, liver mineral.

INTRODUCTION

Sulfur (**S**) is an important element for the ruminant animal due to its role in adequate growth, and in the metabolism of many ruminal bacteria (Drewnoski et al., 2014). Despite its importance, concentrations of S above NASEM (2016) recommendations have been related to reductions on performance, trace mineral absorption, and the incidence of polioencephalomalacia (PEM), with the latter being caused by the reduction of S to H₂S in the rumen and its effect at the cellular level within the brain (Gould, 1998; Drewnoski et al., 2014). Bismuth subsalicylate (**BSS**) has been extensively used in humans to bind to H₂S and, consequently, form bismuth sulfide (**B₂S₃**; Suarez et al., 1998). Using BSS as a ruminal H₂S mitigator has not been extensively evaluated until recently when Ruiz-Moreno et al. (2015) looked at the effects of different concentrations of BSS in vitro. Enteric methane (**CH₄**) generated in the gastrointestinal tract of ruminants is one of the most abundant greenhouse gas (**GHG**) sources that exist in the livestock industry (Caro et al., 2016). Nitrate has been researched in ruminants primarily as a potential CH₄ mitigator due to its H⁺ mechanism that act upon the ruminal environment (Leng, 2008; Newbold., 2014; van Zijderveld et al., 2010).

The combination of BSS and nitrate as an aid to alleviate some of the negative effects caused by S and H₂S has not been extensively investigated (Henry et al., 2016). We hypothesized that by adding nitrate and BSS to the diet of beef cattle, some of the negative effects observed when cattle are consuming high sulfur diet would be reduced, which could improve performance, apparent total tract nutrient, liver mineral absorption, and carcass quality. Therefore, the objective of this experiment was to look at the effects

of BSS and eCAN on beef cattle performance, apparent total tract nutrient digestibility, liver mineral concentration, and carcass characteristics.

MATERIALS AND METHODS

All experimental procedures involving live animals during this study were conducted at the Texas Tech Burnett Research Center located in New Deal, Texas. Animals were handled in compliance with the regulations of the Institutional Animal Care and Use Committee (IACUC protocol #T18079).

Animals

Two hundred and thirty five Angus crossbreed steers ($BW = 385 \pm 27$ kg) were received on December 28, 2018 at the Texas Tech University Burnett Center Feedlot within the New Deal Research and Education Center. After arrival, cattle were allocated to earth floor receiving pens ($n=16$) and were provided with ad libitum access to water, B-dahl hay, and a 65% concentrate receiving diet delivered at 2.5% of incoming BW. Three days after arrival (d -21), all cattle were identified individually with a unique ear tag and treated in accordance with the Texas Tech University protocols. Briefly, the initial processing included the application of an antiviral (Bovi-Shield Gold 5; Zoetis, Florham Park, NJ), anti-clostridial (One-Shot Ultra 7; Zoetis, Florham Park, NJ), and against mycoplasma bovis (Myco-Vac B; Texas Vet Lab Inc., San Angelo, TX), and finally a treatment for internal parasites (Detomax Pour-On; Zoetis, Florham Park, NJ).

Cattle were weighed individually (Silencer Chute; Moly Manufacturing, Lorraine, KS; mounted on Avery Weigh-Tronix load cells, Fairmount, MN, accuracy ± 0.45 kg) (d-14) to be distributed into blocks based on their BW ($n = 10$ blocks; 20 head/block). After being blocked, cattle were returned to the receiving pens and sorted into their respective

blocks two days later (d -12). On d -8, cattle were sorted into 50 concrete, partially slotted-floor pens (4 head/pen; 2.9×5.5 with 2.4 m of linear bunk space). Liver biopsies were collected on d -5 from one animal/pen that was randomly selected previously, on the same day all cattle received an implant with a combination of trenbolone acetate + estradiol benzoate (Synovex Choice; Zoetis, Parsippany, NJ). Four days after liver biopsy extractions, and implantation, cattle were weighed to record IBW (d 0) and started a 4-week adaptation period where the treatments were administered and randomly assigned to each of the 50 pen.

Treatments, Diet, and Experimental Design

For this experiment, five treatments were used in a randomized complete block design with a $2 \times 2 + 1$ factorial arrangement of treatments. The factors were 2 dietary concentrations of BSS (0.0 and 0.33%) and two dietary concentrations of encapsulated calcium-ammonium nitrate (**eCAN**; 0.0 and 2.0%). The concentration of ruminally available sulfur (**RAS**) was 0.28% for the 2×2 portion of the experiment, and 0.14% **RAS** for the + 1 portion. The addition of eCAN on this study is being evaluated as an alternative nonprotein nitrogen source (**NPNS**) replacing urea; therefore, urea was added at 0.70% to the treatments without eCAN making them all isonitrogenous. Treatments are labeled as follows: **CTL** (Urea at 0.70% with 0.28% **RAS**), **NIT** (eCAN at 2.0% with 0.28% **RAS**), **UBS** (Urea at 0.70% and BSS at 0.33% with 0.28% **RAS**), **NITBS** (eCAN at 2.0% and BSS at 0.33% with 0.28% **RAS**), and **LOW-S** (Urea at 0.70% with 0.14% **RAS**) (Table 2.1).

All cattle on trial were adapted to eCAN and urea for 4 weeks through a “4-step” protocol based on a modified version published by Newbold et al. (2014). Throughout the

4 steps, the nonprotein nitrogen (**NPN**) source was gradually added starting from 25% of the total inclusion (0.70% Urea and 2.0% eCAN) to 50%, 75%, and 100% for steps 1, 2, 3, and 4, respectively. During the adaptation, all of the animals were closely monitored for any negative health signs. The addition of BSS started at step 1 of the NPN adaptation where it was added at full inclusion (0.33% of the diet DM). The “step-up” protocol also included the adaptation to concentrate in the diet to reach the typical level of a commercial 90% concentrate ration (Table 1). Diets were balanced to meet or exceed the requirements specified by the NASEM (2016). The non-protein nitrogen sources were added directly into the feed during mixing, however, the BSS treatment was added accordingly to the feed as a premix that was made previously with finely ground corn as a carrier (33.33% BSS and 66.66% corn, DM basis).

Treatments were delivered to each pen once per day through freshly mixed feed. All cattle were fed every morning at 0800h using the clean bunk management technique to minimize refusals. Each treatment was mixed with the diet in a paddle type mixer, transferred to a tractor pulled mixer, and delivered to each pen respectively.

Body weight

The total duration of this feeding trial was 171 d. After finishing the adaptation period, all cattle were weighed prior feed delivery (0700 h) on d 37, 72, 107, 142, and 171. Each pen of cattle was brought up to a pen scale where total weight was taken. After being weighed, all of the cattle were returned to their respective pen to be fed.

Dry Matter Intake

Weekly feed samples were taken from each treatment diet immediately after mixing and before feeding. Subsamples were weighed and then placed in a forced air

drying oven at 100°C for 24 h to determine DM. Another sample of the diet was collected and kept at -20°C for further analysis. Orts were collected weekly (every Saturday prior to feeding at 0700h). All orts were weighed and a sample was extracted to be placed in a forced air dryer at 100°C for 24 h to calculate weekly DMI.

Sample Collections for Total Tract Nutrient Digestibility

Feed and fecal samples were collected for a 7 d period from d 44 to d 51 to determine apparent total-tract digestibility. Throughout the 7 days, fecal samples were collected by hand prior to feeding (0700 h) and in the afternoon (1600 h). Additionally, feed samples were collected by hand immediately after feeding (approximately 0800 h) from each bunk, and were placed into brand new plastic bags. Feed samples were stored at -4°C, and all fecal samples were stored at -20°C for chemical analysis.

Liver Tissue Collection and Rectal Temperature

Liver samples were extracted via biopsy from one randomly selected steer per pen on d -5 and 124 to obtain at initial and final liver mineral concentration. During the procedure, each selected steer was restrained with a hydraulic chute (Silencer Chute; Moly Manufacturing, Lorraine, KS).

The biopsy site was located in the 11th intercostal space where a line from where the tuber coxae and the point of the shoulder intersect; here a 1 cm incision was made using a sterile scalpel. Before performing the incision, the biopsy area was shaved using a disposable razor and sanitized using a solution of water and 7.5% povidone iodine surgical scrub (Betadine, Purdue Products, L.P., Stamford, CT), and with 70% ethanol. After sanitation, a local anesthetic (lidocaine HCl, 20 mg/mL, 8 mL per biopsy) was administered subcutaneously around the biopsy area in a square-shaped pattern (4

injection sites, 2 mL of lidocaine HCl per site. To extract the liver tissue, a sterile 16.5 cm long, 6-mm diameter custom designed trocar and sharpened cannula biopsy instrument (SurgiPro, Minneapolis, MN) was used to collect approximately 0.4 g (as-is) of liver tissue (Chapman et al., 1963). The tissue sample was promptly placed in a properly labeled micro-centrifuge tube and into dry ice for further storage.

Immediately after completing the liver tissue extraction, the incision was cleaned with 70% ethanol, closed with a veterinary tissue adhesive (VetBond, 3M Animal Care Products, St. Paul, MN) and coated with a protective aerosol bandage (AluShield, Neogen Corp, Lexington, KY) to prevent any infection during the healing process. Once the biopsy procedure was performed, the samples were transported to the laboratory and were kept at -20°C for further analysis. All of the animals were closely monitored for 2 to 3 d after the procedure to ensure health and wellness. Initial and final liver mineral concentration was determined using ICP-MS (Animal Health Diagnostic Laboratory, Lansing, MI). Rectal temperature was also measured in all animals on d 124 using a rectal thermometer; the thermometer was allowed approximately 15 s in order to obtain an accurate measurement.

Dietary Nutrient Analysis

All samples collected were processed and analyzed in the Sustainable Ruminant Nutrition Laboratory located in the Animal and Food Sciences Building at Texas Tech University. Feed and fecal samples were placed in forced-air dryer at 55°C for 48 h and 72 h, respectively. After being fully dry, all samples were ground to pass through a 2 mm screen (Willey mill, Thomas Scientific, Swedesboro, NJ). Samples that were collected for digestibility from each pen were composited within pen. Briefly, 10 g of each sample

were placed in a new bag and shaken for proper mixing. Weekly diet samples were composited by treatment and shipped to a commercial laboratory (Dairy One Laboratory, Ithaca, NY) for nutrient analysis. To determine DM and organic matter (**OM**), 0.5 g of sample was placed in hot-weighed crucibles and put in forced-air dryer at 100 °C for 16 h. The same samples were placed in a muffle furnace for 5 h at 550°C to completely combust the organic portion before returning them to an air forced dryer at 100 °C.

For CP analysis, 0.3 g of sample were analyzed in duplicate in a LECO nitrogen/protein analyzer (LECO, St. Joseph, MI). To calculate CP, the concentration of N in each samples was multiplied by 6.25.

Neutral and Acid Detergent Fiber Analysis

To perform this analysis, 0.5 g were used in duplicate from each composite. Each sample was placed into a previously weighed and labeled F57 bag (Ankom Technology, Macedon, NY), and sealed with a heat sealer before placing them in the fiber analyzer (Ankom200, Ankom Technology, Macedon, NY). Sample bags were placed on the machine's suspenders and inserted into the vessel of the fiber analyzer, immediately after that, 1900 mL of ambient temperature neutral detergent solution was poured into the machine for full submersion of the bag suspenders. To ensure starch digestion, 4 mL of heat stable α -amylase was added, and 19 g of sodium sulfite. Samples bags were placed in an air forced dryer at 100 °C for 16 h to be weighed for NDF calculations. The NDF and ADF procedures were performed subsequently on the same samples as described by Van Soest et al. (1991).

Indigestible Neutral Detergent Fiber

Feed and fecal samples were analyzed to determine the concentration of indigestible NDF (**iNDF**; internal marker to calculate apparent nutrient digestibility), with a modification of the procedure by Cole et al. (2011). For this procedure, 0.5 g of sample were weighed into F57 Ankom bags (Ankom Technology, Macedon, NY) in duplicate, these bags were then incubated in the rumen of a cannulated steer consuming grass hay for 288 h to ensure complete degradation of potentially digestible NDF. After incubation, all samples were rinsed twice with tap water followed by 4 rinses with DI water. The rinsed bag samples were ultimately analyzed for NDF as it has been previously described.

Harvest and Carcass Data Collection

Cattle were transported 169 km to a commercial abattoir (Cargill Meat Solutions, Friona, TX) where trained personnel from Texas Tech University collected carcass measurements. Individual carcass measurements included hot carcass weight (**HCW**), fat thickness (**FT**), Longissimus Muscle (**LM**) area, quality grade (**QG**), marbling scores (**MS**), dressing percentage (**DP**), calculated yield grade (**CYG**), and liver scores following the Eli Lilly Check System (Elanco Animal Health, Greenfield, IN) as described by Brown and Lawrence (2010).

Statistical Analysis

Data were analyzed as a randomized complete block design with a $2 \times 2 + 1$ factorial arrangement of treatments. Performance data, rectal temperature, apparent total tract nutrient digestibility, liver mineral concentration, and continuous variables (HCW, FT, LM area, MS, and DP) were analyzed using the MIXED procedure of SAS (SAS

version 9.4; SAS Inst., Cary, NC). Initial liver mineral concentration was used as covariate in order to evaluate final liver mineral concentration. Pen was considered the experimental unit. For continuous data, the models included the fixed effect of treatment and block was considered a random effect. All categorical data (QG, YG, and Liver abscess) were analyzed using the GLIMMIX procedure of SAS (SAS version 9.4; SAS Inst., Cary, NC). For categorical data, the models included the fixed effect of treatment, the random effect of block and pen was considered the experimental unit. Contrast statements were utilized to evaluate the effect of RAS between CTL and LOW-S treatments, as well as the effect of NPN source (**NPNS**), the inclusion of BSS, and the interaction between BSS and NPNS. The Tukey-Kramer adjustment was used to account for multiple comparison and significance was declared at $P \leq 0.05$ and tendencies are discussed at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Effects on Cattle Performance

All growth and performance data are presented on Table 2.2. Final body weight (**FBW**) was affected by NPNS ($P = 0.045$) where cattle consuming urea were 14 kg heavier than cattle consuming eCAN. There was a NPNS effect on DMI ($P = 0.039$) from d 0 to 37 where cattle receiving nitrate had lesser DMI than cattle receiving urea. Additionally, a BSS $\times$ NPNS interaction was observed between d 0 - 37, cattle that received UBS had greater ADG ($P = 0.021$) and G:F ($P = 0.015$) than CTL; however, the opposite occurred for cattle consuming nitrate where ADG and G:F were greater for NIT when compared to NITBS. From d 72 to 107, an NPNS effect was observed for DMI ($P = 0.001$) where cattle receiving CTL and UBS had greater intake than NIT and NITBS

cattle. There was also a NPNS effect on ADG ($P = 0.007$) and DMI ($P < 0.001$) from d 107 to 142 where CTL and UBS cattle had greater ADG and DMI than NIT and NITBS cattle. Additionally, there was a S effect on ADG ($P = 0.021$) and DMI ($P = 0.029$) where CTL animals gained more than LOW-S animals and consumed less; however, no effects were observed on G:F ($P = 0.116$). The reduction of DMI on cattle consuming nitrate can be attributed to a number of factors. For example, when nitrate enters the rumen, nitrite (NO_2^-) is one of the nitrate-reduction intermediaries (Olijhoek et al., 2016). It is possible that when NO_2^- builds up in the ruminal environment to the point that it surpasses the reduction capacity of the microbes, it can be absorbed in the blood and increase the levels of methemoglobin, which triggers a reduction in DMI (Lee et al., 2017). It has also been suggested that nitrate could change the organoleptic properties of the diet when it is added to it, especially palatability, which can explain the reduction in DMI, especially when cattle start consuming the nitrate.

There was an interaction observed for ADG ($P = 0.019$) from d 142 to 171 where CTL cattle had greater ADG than UBS cattle; however, the opposite occurred for eCAN treatments where NITBS had greater ADG than NIT. Non-protein nitrogen source ($P = 0.004$) and S ($P = 0.012$) had an effect on DMI where cattle consuming eCAN had lesser DMI than cattle consuming urea; when CTL and LOW-S were compared, CTL had greater DMI than LOW-S. Even though DMI was affected by NPNS and S, there was only an interaction observed on G:F ($P = 0.018$) between d 142 – d 171 where CTL had greater G:F than UBS and NIT had greater G:F than NITBS as well, which follows the same pattern observed for ADG. In Chapter 3, the effects of calcium-ammonium nitrate (CAN) on VFA molar proportions are discussed where CAN tended to increase acetate

concentration which resulted in a greater A:P, likewise, BSS has been observed to increase acetate production (Ruiz-Moreno et al., 2015; Henry et al., 2016). It is possible that some of the results observed on ADG and G:F could be a response of a shift in VFA profile in the rumen caused by the combination of eCAN and BSS that could have made the usage of nutrients less efficient in terms of energy utilization. Furthermore, these data indicate that the effects observed on ADG and G:F by NPNS may be caused by the reductions on DMI, which differs with previous data where a reduction in DMI has been observed with improvements on ADG, and consequently greater G:F (Newbold et al., 2014; Lee et al., 2017).

When performance was analyzed for the total duration of the trial (d 0 to 171), DMI ($P < 0.001$) and ADG ($P = 0.041$) were affected. Adding eCAN as a NPNS reduced DMI ($P < 0.001$) by 0.50 kg when compared to urea treatments, which represents a 5.43% reduction. Additionally, ADG was reduced ($P = 0.041$) by eCAN compared to urea treatments; however, G:F was not affected ($P = 0.876$). Bismuth showed a tendency to reduce DMI by the culmination of the trial ($P = 0.095$) but did not affect ADG ($P = 0.436$) or G:F ($P = 0.876$). The effect of S was also observed ($P = 0.042$) where LOW-S cattle had lesser DMI than CTL. Lastly, a treatment effect was observed ($P = 0.001$) between CTL and NIT where cattle receiving NIT consumed 6.31% less feed than cattle receiving CTL. However, regardless of the effects observed on DMI for the entire duration of the trial (d 0 – d 171), G:F was not affected by any of the treatments ($P = 0.982$). Effects on performance caused by feeding nitrate to cattle are found in previous data. It has been reported that linear reductions in feed intake as levels of dietary nitrate were increased without affecting ADG (Newbold et al., 2014). In an experiment with 300

Nellore bulls consuming up to 2.4% of the feed DM resulted in a linear increase in G:F by nitrate (Newbold et al., 2014). Similarly, Lee et al. (2017) observed reductions in DMI from cattle consuming nitrate at 2.5% of diet DM with tendencies to increase ADG, which consequently improved G:F. The data presented by previous researchers indicates that reductions on DMI from nitrate are expected with improvements in G:F; however, even though there was a reduction in DMI in the current experiment, G:F was unaffected ($P = 0.876$) since ADG was also reduced.

Effects on Apparent Total Tract Nutrient Digestibility

Apparent total tract digestibility of nutrient data are presented on Table 2.4. There was a tendency for a BSS effect ($P = 0.056$) on DM digestibility (**DMD**) where the addition of BSS tended to increase DMD compared to the non-BSS treatments. There was a BSS effect ($P = 0.045$) on OM digestibility (**OMD**) where treatments with BSS had greater OMD than non-BSS treatments. Henry et al. (2016) observed that adding BSS above 0.33% of the substrate DM can reduce IVOMD linearly; however, the authors did not observed a difference between 0.00 % and 0.33% of the substrate DM. Moreover, it has also been observed that BSS may not affect apparent total tract digestibility of OM (Henry et al., 2018a). It is unclear how BSS may be increasing OMD and DMD. The data observed in the current experiment is not in agreement with previous reports from Henry et al. (2018a); therefore, more research should be done to further study the effect of BSS on DMD and OMD on ruminants.

There was a tendency for an interaction ($P = 0.055$) on CP digestibility (**CPD**) where UBS tended to have greater CPD compared to CTL. A tendency for an interaction ($P = 0.083$) was also observed for ADF digestibility (**ADFD**), NIT tended to have greater

ADFD than NITBS; however, CTL tended to have lesser ADFD than UBS. Ruiz Moreno et al. (2015) evaluated the addition of BSS on in vitro digestibility of OM, NDF, and ADF, using a dual-flow continuous culture system, their data show reductions in OM, NDF and ADF digestibility when BSS was added at 1.0 % of the substrate DM. The results obtained in this experiment contradict most of the previous data on apparent total tract nutrient digestibility when eCAN and BSS are fed to cattle (Henry et al., 2018a). It is important to mention that the research performed with the combination of these compounds has been performed with different types of diets among experiments, which could open the possibility for more investigation in order to elucidate some of the results obtained thus far.

Effects on Carcass Characteristics

There was an effect of NPNS on HCW ($P = 0.032$; Table 2.4) where cattle receiving urea had greater HCW than those receiving eCAN. Previous data show a lack of effect on HCW by nitrate (Newbold et al., 2014). Additionally, other authors have reported data where eCAN have not affected carcass characteristics (Lee et al., 2017); however, Pesta et al. (2016) observed that feeding calcium nitrate to cattle reduced HCW, which is in agreement with our data. Moreover, it is possible that the reduction observed on HCW by eCAN can be related to the decrease in FBW where cattle consuming eCAN were 14 kg lighter than cattle consuming urea. A tendency for a BSS effect ($P = 0.080$) was observed in LM area where BSS treatments had lesser LM area than no BSS treatments. An effect was observed for S in FT ($P = 0.029$) where CTL cattle had lesser FT than LOW-S cattle (Table 2.4). Calculated yield was affected ($P = 0.019$) by an interaction of BSS $\times$ NPNS where treatment UBS had greater CYG than NITBS;

however, CTL and NIT did not differ. This data indicate that there may be an additive effect CYG when BSS and eCAN are combined; however, it is unclear to the author why this effects occurred during the current experiment. There was also an S effect ($P = 0.008$) where LOW-S had greater CYG than CTL. No effects were observed on DP, or MS.

Categorical data for carcass characteristics are presented in Table 2.5. There was a tendency for a BSS effect ($P = 0.099$) on QG for Choice where less cattle tended to grade on this category when BSS was added to the diet. No effects were observed on the other QG. Yield grade 3 had a BSS $\times$ NPNS interaction ($P = 0.022$), there was more cattle that graded on YG3 for NITBS compared to NIT, but the opposite effect is observed for CTL and UBS. A tendency for a S effect ($P = 0.059$) was also observed where more cattle graded on YG3 for CTL compared to LOW-S. A tendency for a BSS $\times$ NPNS interaction ($P = 0.068$) was observed on YG4, when UBS and CTL are compared, more cattle graded on YG4 when BSS was in the diet; however, more cattle graded on YG4 when they received NIT compared to NITBS. Very few researchers have looked at the effects of nitrate in combination with BSS on cattle performance (Henry et al., 2018b). Moreover, the effect of these compounds on carcass characteristics have not been looked at with this level of detail. Henry et al. (2018b) evaluated some carcass quality variables using ultrasound on cattle consuming eCAN and BSS. The authors did not observe any effects on LM area; however, there was an interaction BSS $\times$ NPNS where animals consuming the nitrate-bismuth treatment had less back fat compared to the urea-bismuth treatment, which is in disagreement with the data observed in this experiment. To the author's knowledge, only Henry et al. (2018b) and the current experiment have looked at

the effects of BSS and eCAN on carcass characteristics. More importantly, the diets used between experiments was completely different since cattle were consuming a high concentrate diet in the current experiment, whereas for Henry et al. (2018b), cattle were receiving a high forage diet

Previous data about the effects of S on carcass characteristics has been performed by increasing levels of dietary S with DGS (Klopfenstein et al., 2008; Richter et al., 2012). It is important to consider that the nutritional properties of DGS could be the determining factor that is influencing differences observed on carcass quality.

Furthermore, one has to be cautious when comparing carcass data from experiments performed with DGS and inorganic sources of S, especially when the effects of S are being evaluated. Therefore, the effects observed in the current experiment on carcass quality are still unclear.

No differences were observed on liver scores, however, there was a 5% presence of liver abscesses when cattle consumed the LOW-S treatment compared to a 17.95% from cattle receiving CTL. The incidence of liver abscesses is generally considered to be related to ruminal acidosis and the lesions that a continual high acid load can create on the ruminal wall, which in consequence, leads to the entry of microorganisms into the portal system that can cause liver abscesses (Galyean and Rivera, 2002). An increase in H₂S concentration has been related to reductions in ruminal pH, which is indicative that cattle consuming a finishing ration are more susceptible to greater ruminal concentrations of H₂S (Gould et al., 1997). Additionally, it has been suggested that H₂S may affect smooth muscle in the rumen (Drewnoski et al., 2014). Therefore, it could be possible that the combination of low ruminal pH and greater concentrations of H₂S which

could possible increase the occurrence of liver abscesses in cattle consuming high inclusions of dietary S.

Rectal Temperature

There were no differences observed for rectal temperature ($P > 0.10$) during this trial (Table. 2.7). Henry et al, (2017) reported a reduction on rectal temperature of cattle consuming BSS at 0.33 % of the diet DM compared to those consuming the non-BSS treatment. The author attributed the effect of BSS on rectal temperature to the possibility of BSS acting as a nonsteroidal anti-inflammatory drug (**NSAID**; Henry et al., 2017). Moreover, salicylate has been observed to alter body temperature by inhibiting both COX-1 and COX-2, which consequently decrease prostaglandins that cause inflammatory response (Cashman, 1996). It is important to consider that the effect of BSS on rectal temperature of ruminants has only been observed previously when cattle were consuming a high forage diet Henry et al. (2017), whereas the diet utilized in this experiment was high concentrate. Therefore, it could be possible that diet type is influencing the effects of BSS as a NSAID.

Liver Mineral Concentration

Initial concentration of minerals can be found on Table 2.6. There was a tendency ($P = 0.086$) for an effect of treatment on initial Zn concentration where NITB tended to be different than NIT ($P = 0.059$). All animals were receiving the same diet and liver biopsy samples were collected before treatments were administered, therefore, any effects observed on initial levels of mineral are merely random.

Final liver mineral data are presented on Table 2.7. There was a tendency ($P = 0.099$) for a treatment effect on final Se where CTL tended to have greater concentration

than the rest of the treatments. There was an effect of BSS ($P = 0.042$) in final concentration of Cu where BSS treatments reduced Cu by 58% when compared to the non-BSS treatments. Final concentration of Fe, Zn, and Mn were not affected. It is hypothesized that when BSS enters the rumen, it will bind to S forming Bi_2S_3 ; therefore, reducing the amount of available sulfur that would otherwise bind to trace minerals, and consequently the absorption of these minerals would be greater. The results observed in this experiment show that BSS can reduce the absorption of some minerals such as Cu, which does not support our hypothesis. Henry et al. (2018) observed the same effect on final Cu concentration where BSS reduced the absorption of Cu. It is possible that BSS is not only binding to S, but it is also binding to Cu, forming Bi-Cu-S compounds and reducing the availability of Cu for absorption. The effect of bismuth on mineral retention of rat has been observed previously, where injections of BiCl_3 showed an increase in total body Cu content with no effects on liver Cu concentration (Szymanska and Zelazowski, 1979). The authors observed that while concentration of Cu was not affected in the liver, kidney concentrations of Cu were increased by bismuth (Szymanska and Zelazowski, 1979). It can be speculated that bismuth may bind to Cu, and once this occurs, Cu becomes unavailable for liver storage and it is excreted through the urine; however, to the author's knowledge, there is no data reported on Cu urinary excretion of cattle or any species consuming bismuth. Henry et al. (2018b) observed an increase on Fe concentration in cattle consuming BSS. The authors attributed this effect to the reduction of Cu absorption since Fe is mobilized by ferroxidase, a Cu dependent enzyme (Henry et al., 2018b; Suttle, 2010); however, the data observed in this experiment did not show the same response on Fe, where the concentration of this mineral in the liver was not affected

by BSS ($P = 0.455$). Henry et al. (2018b) reported a 70% reduction in Cu concentration by BSS, whereas in the current experiment, BSS reduced final Cu by 58%; therefore, it is possible that the amount of Cu depleted in the current experiment was not enough to cause a response in final Fe.

Changes in liver mineral concentration can be found on Table 2.8. There was a tendency ($P = 0.059$) for a NPNS effect on mineral concentration change of Se where nitrate treatments tended to have a greater change in Se concentration. A tendency ($P = 0.073$) for a BSS effect was also observed on Cu concentration change where BSS treatments tended to increase the change in Cu concentration within the liver. Change in Zn concentration was affected by BSS ($P = 0.025$) where BSS had showed a greater change when compared to non-BSS treatments. Change in concentration of Fe, Mn were not affected ($P > 0.10$). When the change in mineral concentrations was analyzed, the tendency for a treatment effect ($P = 0.099$) observed on final concentration of Se became a tendency for a NPNS effect where nitrate increased the change in Se concentration. Unfortunately, to the author's knowledge, few researchers have reported data where the change in mineral concentration has been evaluated when cattle received nitrate. It is unclear to the author why nitrate had a tendency to affect change in concentration of Se. The effect of BSS ($P = 0.025$) on Zn concentration change may be attributed to the effect of BSS on initial concentration of Zn ($P = 0.015$), which as discussed previously, was due to random variation. Finally, the tendency for an effect of BSS on Cu concentration change is a response to the main effect of BSS observed on final Cu concentration which showed a 58% reduction of Cu in the liver. Prados et al. (2017) reported that adequate concentrations of liver Cu are from 100 to 400 mg/Kg. During the current experiment,

cattle consuming BSS had a final concentration of Cu of 47.3 mg/kg within the liver, which indicates that cattle had a deficiency of Cu; however, the performance of these cattle was not affected and there were no symptoms of a Cu deficiency detected. Furthermore, despite the lack of Cu deficiency symptoms, a dietary source of Cu should be included in the diet if BSS is added to avoid any potential deficiencies.

CONCLUSIONS

Nitrate has been observed to reduced DMI while improving ADG and G:F in cattle; however, the current experiment showed a different outcome where nitrate did not improve ADG and G:F. Some of the effects observed by nitrate on performance, especially during the first weeks of the experiment indicate that a shift in microbial fermentation could be happening potentially due to the adaptation of the rumen microorganisms to nitrate. Some of the effects observed on carcass quality are still unclear to the author. The majority of the data on carcass quality response to high levels of S has been performed with DGS, therefore, caution must be taken when comparing results among experiment when inorganic sources of S are used. The response of Cu concentration in the liver could be a result of BSS binding to S and Cu creating Bi-Cu-S compounds that make Cu less available for liver storage. Although the final levels of Cu in the liver did not affect animal performance, it may be ideal to add a source of Cu in the diet when BSS is being included to avoid any deficiencies. In conclusion, even though some of the effects caused by eCAN and BSS has not been observed previously, more research should be performed to look at the effects within the rumen when this treatments are fed to cattle. Perhaps, analyzing the effects in the rumen could help elucidate some of the results observed in the feedlot. Finally, even though there was not an statistical

difference in liver abscess incidence between CTL and LOW-S, the 12.95 percentage units difference is may be an indicative that S level could be affecting liver abscess occurrence; therefore, more research should be performed to elucidate these results.

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TABLES**Table 2. 1.** Description of the basal diet (DM basis)¹

Item	Treatments ²				
	CTL	NIT	UBS	NITBS	LOW-S
Ingredients, % DM					
Steam-flaked corn	61.27	61.00	61.27	61.27	61.52
Wet corn gluten feed	20.00	20.00	20.00	20.00	20.00
Cottonseed, hulls	5.00	5.00	5.00	5.00	5.00
Alfalfa hay, chopped	5.00	5.00	5.00	5.00	5.00
Fat (yellow grease)	3.00	3.00	3.00	3.00	3.00
Mineral supplement	2.00	2.00	2.00	2.00	2.00
Limestone	1.30	0.20	1.30	1.30	1.80
Ground corn	1.00	1.00	1.00	1.00	1.00
Urea	0.70	—	0.70	—	0.70
eCAN	—	2.00	—	2.00	—
BSS	—	—	0.33	0.33	—
Calcium sulfate	0.75	0.75	0.75	0.75	—
Nutrient Composition, % DM ³					
DM	77.88	77.29	77.75	77.63	77.88
CP	13.40	13.20	13.50	13.60	13.40
OM	94.42	94.81	94.16	94.26	94.53
NDF	17.90	14.50	18.60	19.40	18.20
ADF	8.90	6.80	7.70	6.60	9.50
TDN, %	76.00	78.00	76.00	76.00	76.00
NE _m , Mcal/kg	1.87	1.91	1.86	1.86	1.87
NE _g , Mcal/kg	1.24	1.27	1.23	1.22	1.23
Ca	0.73	0.78	0.77	0.80	0.78
P	0.50	0.52	0.52	0.54	0.53
Mg	0.21	0.21	0.21	0.22	0.22
K	1.04	1.04	1.02	1.10	1.09
Na	0.18	0.19	0.18	0.20	0.20
S	0.32	0.33	0.34	0.37	0.27
Fe, ppm	234.00	212.00	214.00	212.00	223.00
Zn, ppm	94.00	106.00	94.00	108.00	109.00
Cu, ppm	11.00	13.00	12.00	14.00	12.00
Mn, ppm	30.00	33.00	33.00	33.00	37.00

¹Diet was formulated to meet or exceed NRC (2016) requirements for finishing beef cattle.²CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).³Chemical composition values were calculated through proximate analysis (Dairy One Laboratory, Ithaca, NY).

Table 2. 2. Effect of bismuth subsalicylate and calcium ammonium nitrate on feedlot growth performance

Item ⁴	Treatments ¹						P-value ²				
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
IBW, kg	385	384	384	387	387	8.3	0.194	0.212	0.338	0.342	0.125
FBW, kg	665	644	654	647	657	10.0	0.217	0.380	0.045	0.531	0.301
d 0-37											
ADG, kg	2.0 ^{ab}	2.0 ^{ab}	2.2 ^b	1.8 ^a	2.0 ^{ab}	0.08	0.035	0.832	0.016	1.000	0.021
DMI, kg	8.09	7.91	7.94	7.86	7.95	0.081	0.126	0.121	0.039	0.120	0.441
G:F	0.250 ^{ab}	0.252 ^{ab}	0.276 ^b	0.233 ^a	0.257 ^{ab}	0.0087	0.028	0.577	0.025	0.693	0.015
d 37-72											
ADG, kg	1.6	1.7	1.7	1.9	1.7	0.09	0.354	0.510	0.186	0.224	0.284
DMI, kg	8.76	8.52	8.74	8.57	8.72	0.285	0.969	0.918	0.497	0.969	0.907
G:F	0.193	0.200	0.192	0.218	0.204	0.0132	0.678	0.577	0.240	0.542	0.500
d 72-107											
ADG, kg	1.9	1.8	1.8	1.7	1.8	0.12	0.610	0.427	0.471	0.148	0.975
DMI, kg	10.47 ^c	9.67 ^{ab}	9.99 ^{abc}	9.54 ^a	10.35 ^{bc}	0.213	0.001	0.638	0.001	0.075	0.357
G:F	0.184	0.192	0.176	0.176	0.173	0.0109	0.638	0.428	0.683	0.224	0.683
d 107-142											
ADG, kg	1.4 ^b	1.2 ^a	1.4 ^b	1.2 ^a	1.2 ^a	0.07	0.044	0.021	0.007	0.954	0.882
DMI, kg	10.44 ^c	9.33 ^{ab}	10.00 ^{bc}	9.10 ^a	9.75 ^{abc}	0.228	<0.001	0.029	<0.001	0.126	0.623
G:F	0.136	0.127	0.412	0.131	0.118	0.0077	0.281	0.116	0.214	0.531	0.900
d 142-171											
ADG, kg	1.0	0.74	0.68	0.87	1.0	0.10	0.058	0.828	0.601	0.256	0.019
DMI, kg	9.00 ^b	8.40 ^{ab}	8.65 ^{ab}	8.33 ^a	8.44 ^{ab}	0.146	0.022	0.012	0.004	0.171	0.358
G:F	0.116 ^{bc}	0.090 ^a	0.076 ^a	0.104 ^b	0.120 ^c	0.0116	0.041	0.800	0.964	0.266	0.018
d 0-171											
ADG, kg	1.64	1.52	1.58	1.52	1.58	0.04	0.247	0.287	0.041	0.436	0.480
DMI, kg	9.35 ^b	8.76 ^a	9.07 ^{ab}	8.66 ^a	9.02 ^{ab}	0.116	0.001	0.042	<0.001	0.095	0.390
G:F	0.174	0.173	0.173	0.175	0.175	0.0033	0.982	0.825	0.876	0.876	0.640

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS. Means with different letters are significant ($P \leq 0.05$).

³ n = 10 experimental units/treatment mean.

⁴Item= IBW= Initial body weight; FBW= Final body weight; d= day; ADG= Average daily gain (kg/day); DMI= Dry matter intake (kg/day); G:F= Gain to feed ratio.

Table 2. 3. Effect of bismuth subsalicylate and calcium ammonium nitrate on nutrient digestibility

Item ⁴	Treatments ¹						<i>P</i> -value ²				
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
DM digestibility, %	65.96	67.73	72.35	68.87	64.49	2.532	0.060	0.590	0.657	0.056	0.176
OM digestibility, %	68.12	69.43	74.13	70.96	66.74	2.421	0.063	0.593	0.611	0.045	0.225
CP digestibility, %	53.70	55.88	63.97	55.66	51.69	3.464	0.026	0.593	0.253	0.065	0.055
NDF digestibility, %	26.26	32.81	31.68	27.66	21.19	6.006	0.370	0.436	0.770	0.975	0.227
ADF digestibility, %	18.69	26.33	27.92	19.30	15.86	6.431	0.304	0.692	0.914	0.812	0.083

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³ n = 10 experimental units/treatment mean.

⁴Item= DM= Dry matter; OM= Organic matter; CP= Crude protein; NDF= Neutral detergent fiber; ADF= Acid detergent fiber.

Table 2. 4. Effect of bismuth subsalicylate and calcium ammonium nitrate on carcass characteristics

Item ⁴	Treatments ¹						P-value ²				
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
Hot carcass weight, kg	413.77	403.79	412.07	403.84	411.48	7.288	0.262	0.694	0.032	0.841	0.831
Dressing, %	62.10	62.50	62.90	62.40	62.60	0.337	0.434	0.239	0.867	0.244	0.136
LM area, cm ²	88.97	86.11	83.88	85.42	86.18	1.949	0.280	0.226	0.685	0.080	0.179
Fat thickness, cm	1.71	1.67	1.78	1.66	1.96	0.076	0.051	0.029	0.282	0.712	0.637
Marbling score	579.14	578.76	583.46	566.09	589.75	17.536	0.916	0.676	0.622	0.816	0.636
Calculated yield grade	3.24 ^{ab}	3.28 ^{ab}	3.68 ^b	3.17 ^a	3.58 ^{ab}	0.121	0.008	0.036	0.044	0.143	0.019

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \leq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³ n = 10 experimental units/treatment mean.

⁴Item= LM= Longissimus muscle area (cm²). Marbling score= 400=Small⁰⁰; 500= Modest⁰⁰; 600= Moderate⁰⁰.

^{a,b,c}Means with different letters are significant ($P \leq 0.05$).

Table 2. 5. Effect of bismuth subsalicylate and calcium ammonium nitrate on categorical carcass traits

Item ⁴	Treatments ¹					P-value ²					
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
Quality grade, %											
Prime	7.70	14.29	17.95	10.26	19.44	6.301	0.513	0.137	0.964	0.551	0.177
Premium Choice	61.54	60.00	56.41	56.41	66.67	8.046	0.742	0.409	0.571	0.908	0.446
Choice	29.03	25.64	15.38	15.38	19.44	8.469	0.568	0.385	0.843	0.099	0.843
Select	0.00	0.00	6.45	7.69	5.56	3.767	0.996	0.979	1.000	0.970	1.000
Yield grade, %											
1	0.00	0.00	0.00	0.00	0.00	0.000	1.000	0.960	0.989	0.994	0.999
2	5.13	14.29	5.13	17.95	11.11	5.968	0.303	0.342	0.038	0.818	0.818
3	64.10	45.71	38.46	53.85	44.44	7.233	0.119	0.059	0.827	0.217	0.022
4	23.08	38.46	41.03	28.21	36.11	8.017	0.437	0.226	0.817	0.592	0.068
5	0.00	0.00	11.43	0.00	10.00	3.470	0.999	0.971	0.983	0.983	0.983
Liver score, %											
Edible	82.05	89.74	87.18	82.05	95.00	6.449	0.479	0.112	0.796	0.796	0.287
Abnormal	17.95	10.26	12.82	17.95	5.00	6.449	0.479	0.112	0.796	0.796	0.287

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³The highest SEM was included in this table.

⁴Item= Liver scores= Edible= No presence of abscess; Abnormal= at least one abscess.

Table 2. 6. Initial levels of mineral in the liver

Item	Treatments ¹							<i>P</i> -value ²			
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
Initial liver mineral, mg/kg DM											
Se	2.12	2.20	2.08	2.34	2.31	0.133	0.533	0.284	0.192	0.691	0.505
Fe	329.73	277.59	341.04	346.99	240.00	34.778	0.179	0.083	0.529	0.274	0.430
Cu	263.50	269.33	239.32	305.39	302.83	31.518	0.478	0.342	0.235	0.843	0.318
Zn	127.64	118.90	138.40	156.03	134.84	9.498	0.086	0.580	0.638	0.150	0.168
Mn	9.33	9.38	9.38	9.89	9.49	0.524	0.944	0.829	0.605	0.609	0.673

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³The highest SEM was included in this table.

Table 2. 7. Effect of bismuth subsalicylate and calcium ammonium nitrate on final liver mineral concentration

Item	Treatments ¹								<i>P</i> -value ²		
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
Final liver mineral ⁴ , mg/kg DM											
Se	1.81	1.27	1.28	1.27	1.68	0.193	0.099	0.619	0.149	0.156	0.160
Fe	397.91	298.65	400.27	372.50	407.76	54.277	0.537	0.211	0.211	0.455	0.479
Cu	134.35 ^{ab}	89.95 ^{ab}	61.34 ^{ab}	33.26 ^a	171.98 ^b	30.566	0.019	0.384	0.251	0.042	0.794
Zn	133.99	114.17	103.89	105.18	112.87	12.878	0.443	0.218	0.454	0.145	0.403
Mn	10.71	7.93	7.96	8.11	9.25	0.923	0.125	0.233	0.142	0.153	0.103
Rectal temperature, °C	38.93	39.09	38.91	39.00	39.04	0.081	0.523	0.356	0.132	0.536	0.736

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \geq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³The highest SEM was included in this table.

⁴Final liver mineral was analyzed using initial liver mineral concentration as a covariate.

Table 2. 8. Effect of bismuth subsalicylate and calcium ammonium nitrate on liver mineral concentration change

Item	Treatments ¹							<i>P</i> -value ²			
	CTL	NIT	UBS	NITBS	LOW-S	SEM ³	TRT	S	NPNS	BSS	BSS × NPNS
Liver mineral change ⁴ , mg/kg DM											
Se	-0.31	-0.93	-0.81	-1.06	-0.62	0.225	0.150	0.322	0.059	0.161	0.421
Fe	62.84	30.64	33.67	16.00	184.02	67.689	0.399	0.207	0.717	0.750	0.916
Cu	-131.81	-180.81	-186.72	-266.32	-125.55	38.894	0.075	0.906	0.100	0.073	0.690
Zn	2.00	-13.66	-32.67	-39.41	-22.31	13.010	0.185	0.181	0.391	0.025	0.732
Mn	1.42	-1.32	-1.36	-1.89	-0.24	1.123	0.250	0.293	0.156	0.148	0.333

¹CTL= Control (Urea at 0.70% with 0.28% RAS); NIT= Nitrate (eCAN at 2.0% with 0.28% RAS); UBS= Urea-Bismuth (Urea at 0.7% and BSS at 0.33% with 0.28% RAS); NITBS= Nitrate-Bismuth (eCAN at 2.0% and BSS at 0.33% with 0.28% RAS); LOW-S= Low sulfur (Urea at 0.70% with 0.14% RAS).

²Significance is declared at $P \leq 0.05$, tendencies are discussed at $0.05 > P \leq 0.10$. TRT= Effect of treatment; S= Effect of sulfur; NPNS= Effect of non-protein nitrogen source; BSS= Effect of BSS; BSS × NPNS= Interaction between NPNS and BSS.

³The highest SEM was included in this table.

⁴Change in liver mineral status was calculated by subtracting final mineral concentration minus initial mineral concentration.

CHAPTER V

INTEGRATED SUMMARY

Sulfur is an important element for the ruminant animal, this mineral occurs in essential amino acids such as cysteine, cystine, and methionine, and it is also important for the metabolism of a number of ruminal microorganisms. The effects of S on cattle health and performance have become a concern after the cattle industry started utilizing co-products from the ethanol sector to feed cattle. These coproducts carry levels of sulfur that can surpass the requirements of beef cattle, which can negatively affect performance and health in the feedlot. When cattle consume diets with high levels of dietary S, H_2S occurs in the rumen that can enter the bloodstream after being inhaled, and can cause cellular necrosis in the brain, resulting in the expression of PEM. Additionally, dry matter intake can be reduced due to the discomfort created by high concentrations of H_2S within rumen, and S can act as a trace mineral antagonist, which can create deficiencies. Many strategies have been investigated to combat the negative effects of S in beef cattle production.

Nitrate has been primarily investigated as a CH_4 mitigator in ruminants. When nitrate enters the rumen, it acts as a H_2 sink, and reduces the substrate availability for methanogenesis. Bismuth subsalicylate has been observed to be an effective H_2S mitigator when it has been provided to humans and rat. The results observed during the current study in in vitro ruminal fermentation indicate that bismuth subsalicylate and nitrate can be added in the diet of ruminants without affecting ruminal fermentation. During the in vivo portion of this study, nitrate showed a reduction of dry matter intake and average daily gain; however, feed efficiency was not affected. Final body weight was

reduced by nitrate and as a result, hot carcass weight was also reduced. Digestibility of nutrient was only affected for dry matter where bismuth tended to increased dry matter digestibility. The data observed in this experiment also indicate that bismuth reduces the absorption of Cu; therefore, it is recommended to supplement cattle with Cu when bismuth is being administered. Some of the results observed in this study are in disagreement with previous data, therefore, more research should be performed to evaluate the effects of nitrate and bismuth subsalicylate within the rumen in order to elucidate some of the results observed in the feedlot.