

**SUPPLEMENTATION STRATEGIES TO IMPROVE EFFICIENCY OF
FORAGE UTILIZATION AND MITIGATE ENTERIC METHANE
PRODUCTION IN *BOS INDICUS* AND *BOS TAURUS* CATTLE**

A Dissertation

by

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ABSTRACT

Multiple investigations were undertaken to determine the effect of various strategies for improving forage intake, digestion, and ruminal fermentation parameters in *Bos indicus* and *Bos taurus* steers. Protein supplementation strategies and monensin inclusion have the potential to improve animal production efficiency while mitigating enteric CH₄ production; reducing the economic and environmental impact of cattle production systems. Effects of different levels and sources of supplemental protein on ruminal methane-producing activity, rate of NH₃ production, and concentrations of methanogens, protozoa, amino acid-utilizing, and trypticase-metabolizing bacteria were investigated. Subspecies differences were not observed for any of the measured parameters ($P \geq 0.28$). Additionally, the effects of monensin inclusion and withdrawal on intake, digestion, and ruminal fermentation parameters were investigated. During the monensin inclusion phase, a subspecies difference was observed ($P = 0.04$) for total digestible OM intake, and a tendency ($P = 0.07$) for a subspecies difference was observed for CH₄-producing activity. During the monensin withdrawal phase, subspecies differences were observed ($P \leq 0.01$) for total digestible OM intake and OM digestibility, and a tendency ($P \leq 0.09$) for subspecies differences were observed for pH and forage OM intake. Overall, results suggest that the difference in ability of Bi and Bt cattle to utilize forage is diet dependent and may be altered by protein supplementation and monensin inclusion to improve production efficiency and mitigate enteric CH₄ production.

DEDICATION

To my husband Roger and my children Roan and Adelina. I couldn't have done this
without your love and support.

1 Corinthians 13:4-8

⁴Love is patient, love is kind. It does not envy, it does not boast, it is not proud. ⁵It is not
rude, it is not self-seeking, it is not easily angered, it keeps no record of wrongs. ⁶Love
does not delight in evil but rejoices with the truth. ⁷It always protects, always trusts,
always hopes, always perseveres. ⁸Love never fails...

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1 Peter 3:7 says "...husbands, live with your wives in an understanding way..."

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CHAPTER I

INTRODUCTION

The symbiotic relationship between ruminant animals and their resident ruminal microbial population allows them to effectively utilize fibrous (e.g., cellulosic) materials that cannot be utilized by humans (Hungate, 1966). Accordingly, cattle producers utilize forage as a major source of nutrients, but grazing or forage-fed cattle often consume low-quality forage (LQF; $\leq 7\%$ CP) because cattle are produced in areas where it is abundant. Early in the growing season forage quality is generally sufficient to meet cattle nutrient requirements; however, as forages mature, they increase in indigestible fiber content and decrease in CP % and digestibility. Providing supplements or feed additives to ruminant animals consuming LQF provides an opportunity to stimulate forage intake and digestion, optimizing the utilization of these forages and efficiency of beef production.

Ruminant livestock are one of the few natural sources of CH₄ emissions that can be manipulated efficaciously (Shibata and Terada, 2010) and, as such, have been identified as an avenue to reduce global CH₄ emissions (Guan et al., 2006). As an added advantage, reduction in CH₄ production improves animal production efficiency. Fermentation of LQF yields greater CH₄ losses (Mcal/d) than does fermentation of higher quality forages (Moe and Tyrrell, 1979), thus the development of technologies allowing more efficient use of LQF is crucial, not only for increased efficiency in livestock production but also for reducing greenhouse gas emissions.

Degradable intake protein is considered to be the first limiting nutrient for microbial growth in the rumen of cattle consuming LQF diets (Cochran et al., 1998). Therefore, protein supplementation commonly promotes microbial cell growth and allows rumen microbes to more effectively degrade available forage. Increased fiber digestion promotes increased forage intake and reduces CH₄ production, permitting more efficient utilization of LQF.

A variety of feed additives have been investigated to improve LQF utilization and increase overall production efficiency. Carboxylic polyether ionophores are a class of feed additives researched in depth (Thornton and Owens, 1981; Wedegaertner and Johnson, 1983; Guan et al., 2006). Ionophores provide an effective means of manipulating ruminal fermentation to improve feed efficiency by decreasing enteric CH₄ emissions. Ready availability of ionophores in the United States, their low cost, and high return make them an ideal method for increasing LQF utilization.

Bos indicus (Bi) and *Bos taurus* (Bt) cattle are commonly fed in the U.S., with Bt producing more tender carcasses with greater marbling (Crouse et al., 1993). Comparative studies of the digestive functions of Bi and Bt cattle have been conducted to determine the specific mechanism that enables Bi cattle to better utilize nutritional components from LQF than Bt cattle (Howes et al., 1963). It was unknown if responses between the subspecies to supplemental protein or ionophores when provided to a basal diet of LQF were different. Because the activity and composition of the rumen microbial population determines the quantity, quality and array of nutrients available to the host animal, a description of the microbial populations present in the rumens of each

subspecies may help to explain the difference in ability of these subspecies to utilize LQF. The purpose of the proposed research is to determine the effects of various protein supplementation regimens and ionophore inclusion on intake, digestion, ruminal fermentation, and microbial populations in cattle consuming LQF based diets.

CHAPTER II

LITERATURE REVIEW

Ionophores: Mechanisms of action and effects on ruminant production

Overview

Carboxylic polyether ionophores are added to ruminant diets as a strategy for improving animal production efficiency and mitigating enteric CH₄ production. This antimicrobial targets and kills specific ruminal bacteria, changing the microbial ecology (microbiome) of the rumen. In general, gram positive bacteria, such as H₂- and NH₃-producing species, are targeted by ionophores, altering ruminal fermentation. Ionophore induced rumen fermentation changes cause a decrease in: CH₄ production, degradation of protein to NH₃, lactate production and the acetate:propionate ratio (VFA which are indicative of the energy provided to the host animal). Changes in rumen fermentation improve host animal production efficiency by decreasing dry matter intake while maintaining similar rates of live weight gain; this directly impacts profitability for the producer. Some bacteria, however, are not intrinsically sensitive to ionophores. Because populations of resistant bacteria exist in cattle not consuming ionophores, resistance appears to be due to physiological selection rather than a mutation. Thus, use of ionophores in animal feed will not likely have a significant impact on transfer of antibiotic resistance to humans. Ionophore rotation programs have been investigated as a means to alleviate long-term loss of effectiveness of a single ionophore, however, due to inconsistent results, the extent and mode of action of the synergistic benefits remain

unclear. The sustained contribution of ionophores (more than 35 years) to production improvement and enteric CH₄ mitigation support the notion that ionophores will continue to be associated with improved, more efficient, environmentally friendly cattle production systems.

Introduction

The cattle industry relies on innovative management strategies and technologies, such as feed additives, to improve animal health, performance, and profitability. Although a variety of feed additives have been developed through the years, one particular class, ionophores, is capable of producing improvements evident on a global scale. Ruminant nutritionists historically focused on feeding ionophores to improve animal health and efficiency. The negative attention ruminant livestock production receives for enteric CH₄ production's impact on greenhouse gas emissions, however, has shifted the focus to their ability to reduce enteric CH₄ production.

Within a few years of the 1975 FDA approval of monensin as a cattle feed additive, most feedlot cattle were being fed this ionophore. Since that time, the FDA has approved the use of two other ionophores, lasalocid and laidlomycin propionate, as cattle feed additives. Used extensively in feedlots, stocker operations, and replacement heifer operations, ionophores are used commercially throughout the world in all segments of the beef industry. According to the USDA Animal and Plant Health Inspection Service, 90.1% of cattle in U.S. feedlots received ionophores in 2011 (USDA, 2011). Ionophores' ability to improve feed efficiency by 6-8% in feedlot cattle and 8-12% in stocker cattle

was calculated to provide an economic benefit to the producer of approximately \$12/head (Elam and Preston, 2004).

Although not prescribed to treat human bacterial infections, concern has developed that feeding ionophores may pose a threat to public health with regard to antibiotic resistance. However, it is important to question whether rumen antimicrobial resistance is intrinsic or a novel emergence, a topic which, until recently, was not well investigated. Objectives of this review are to thoroughly examine ionophores as CH₄ mitigators and growth promotants as well as impacts of resistance on human health.

Mechanism of ionophore action at the bacterial cell level

Ionophore induced improvements in animal performance are a secondary effect caused by the disruption of normal bacterial membrane physiology (Bergen and Bates, 1984). Bacterial cell membranes are relatively impermeable to ions; accordingly ionic gradients are used to facilitate nutrient uptake at minimal ATP cost (Rosen, 1986). Ionophores carry ions across bacterial cell membranes resulting in death of targeted species. Ionophore application targets changes in rumen bacterial populations, potentially leading to reduced CH₄ emission and consequently improved production efficiency (Schelling, 1984).

Rumen bacteria maintain high intracellular K⁺ (~ 600 mM) and low intracellular Na⁺ concentrations (~ 20 mM; T. R. Callaway, USDA Southern Plains Agricultural Research Center, College Station, Texas, personal communication). Conversely, the ruminal environment is characterized by high Na⁺ (~ 200 mM) and relatively low K⁺ concentrations (~ 10 mM; T. R. Callaway, USDA Southern Plains Agricultural Research

Center, College Station, Texas, personal communication). Ruminant bacteria maintain K^+ and Na^+ gradients to facilitate nutrient uptake.

Ionophores have a hydrophobic exterior and hydrophilic interior, allowing them to bind cations (Russell and Strobel, 1989) forming lipid-soluble complexes with K^+ , Na^+ , Ca^{2+} , Mg^{2+} , and biogenic amines (Pressman, 1976). Due to this interaction and their hydrophobic nature, ionophores are able to shield and delocalize the charge of ions, facilitating the movement of cations across biological membranes (Ovchinnikov, 1979; Russell and Strobel, 1989), disrupting crucial ion gradients (Pressman, 1976).

Ionophores are classified by method of membrane transport as carriers or channel formers. An ion carrier binds to ions acting as a mobile carrier, escorting them through the cell membrane's hydrophobic environment. Ion channels form pores in the membrane allowing ions to pass. Transport cycles across biological membranes can reach thousands of cycles per second (Pressman, 1976) for mobile carriers such as monensin, lasalocid and valinomycin. Other ionophores, such as gramicidin, which act as channel formers spanning the cell membrane, have lower cationic specificity (Russell and Strobel, 1989). While channel forming ionophores are capable of translocating ions at a faster rate than mobile carriers, they have not been used as ruminal feed additives (Russell and Strobel, 1989).

Ionophores are further described by the number of cations they are able to transport. Some ionophores (valinomycin and gramicidin) act as uniporters, transporting only a single cation at a time, while others (monensin and lasalocid) act as antiporters, transporting more than one cation simultaneously (Russell and Strobel, 1989).

Ionophores differ in their affinities for cations; the most common ionophore used in cattle diets, monensin, has an affinity for Na^+ that is ten times greater than for K^+ allowing it to primarily mediate $\text{Na}^+ - \text{H}^+$ exchange due to the large Na^+ and pH gradients maintained by rumen bacteria (Pressman, 1976). Ruminal pH is mildly acidic (ranging from 5.5-6.8, under most conditions; Cunningham, 2002) while intracellular pH is near neutral creating an inwardly directed proton (or pH) gradient. Lasalocid, another ionophore common in cattle diets, has a greater affinity for K^+ . Ionophoric modes of action are explained by their differing affinities for cations, the presence and degree of ion gradients, and interference with normal ion flux through interaction of the ionophore with biological membranes and gradients.

Monensin is the most extensively-investigated ionophore used in ruminant rations; this coupled with its inclusion in >80% of feedlot cattle rations, has resulted in its development as the most complete model of ionophore mode of action (Fig. A1). Monensin is a metal/proton antiporter that exchanges H^+ for either Na^+ or K^+ (Russell and Strobel, 1989). In the rumen environment monensin mediates the exchange of intracellular K^+ ions for extracellular protons, and extracellular Na^+ ions for intracellular H^+ (Callaway et al., 2003). Potassium is the predominant intracellular cation of microorganisms; however, the extracellular K^+ concentration is typically 5 to 15 fold lower than Na^+ . It follows, the K^+ gradient is greater in magnitude than the Na^+ gradient. Monensin is an antiporter, thus, K^+ is exported resulting in the net accumulation of protons inside the bacterium. In a futile effort to maintain ionic equilibrium and/or pH neutrality within the cell, it activates a reversible F_1F_0 ATPase to pump protons from the

cell. Hydrogen ions are exported by facilitated diffusion via an exchange of Na^+ or by active transport involving ATP (Grooms, 2010). Active transport results in an energy expenditure depleting intracellular ATP pools resulting in reduced growth and eventually cell death.

Bacterial membrane physiology and sensitivity

Ionophores are generally most effective against gram-positive bacteria. Gram-positive bacteria have a cell membrane surrounded by a porous peptidoglycan layer allowing small molecules such as ionophores (molecular weight 200-2000; Pressman, 1976) to pass through and dissolve into the lipid bilayer membrane. In contrast, gram-negative bacteria are surrounded by a lipopolysaccharide layer, an outer membrane, a relatively thin peptidoglycan layer and periplasmic space (Callaway et al., 2003) resulting in a relatively impermeable (to lipophilic molecules) structure. Although ionophores are capable of binding to both gram-positive and gram-negative bacteria, CH_4 -mitigation has been associated primarily to their effects on gram-positive species (Bergen and Bates, 1984). Ionophore inclusion has been suggested to provide a competitive advantage to gram-negative bacteria by inhibiting ionophore-sensitive gram-positive species (Chen and Wolin, 1979, Newbold et al., 1993). Limited data using pyrosequencing and other next generation technologies have confirmed this hypothesis (Kim et al., 2014).

Most NH_3 , acetate, butyrate, and hydrogen-producing bacteria are gram-positive species. Because of their sensitivity to ionophores, ruminal populations of these species are generally reduced with dietary inclusion, resulting in reduced concentrations of their

products (NH₃, acetate, butyrate, and hydrogen). Inhibition of the gram-positive bacteria responsible for producing hydrogen indirectly reduces methanogenesis (Czerkawski et al., 1972, van Nevel and Demeyer, 1977; Dellinger and Ferry, 1984). Ruminal CH₄ production rates are correlated (Hungate, 1967; Moss et al., 2000) with dissolved hydrogen concentration in ruminal fluid, as CH₄ is the primary sink for hydrogen (Wolin et al., 1997). Additionally, many ruminal acetate-producing bacteria are sensitive to ionophores. A decrease in acetate production reduced CH₄ production because of its connection to the disposal of reducing equivalents (Hegarty, 1999). Some acetogenic bacteria produce formate (which can be used as a substrate by methanogens), further limiting substrate availability to the methanogen population (van Nevel and Demeyer, 1977).

Effect of ionophores at the animal level

Ionophore use is not only economically justifiable on the farm-scale, but reduced CH₄ emissions further provide global incentive for ionophore usage. Improved production efficiency resulting from inclusion of ionophores into ruminant diets is directly related to: reductions in 1) hydrogen-producing bacteria, 2) NH₃-producing bacteria, 3) lactic acid-producing bacteria and intake as well as 4) changes in energy partitioning and gain.

Impact on hydrogen-producing bacteria

Methane production effects. Ionophores such as monensin, lasalocid, laidlomycin, and salinomycin reduce methanogenesis by inhibiting hydrogen-producing bacteria, a precursor of CH₄ (van Nevel and Demeyer, 1977). Methanogens from the domain *Archaea*, have high affinities for hydrogen and are able to remove much of the hydrogen

from the rumen. Hydrogen-producing bacteria are often gram-positive species which ionophores are most effective against. Concentration of hydrogen-producing species in the rumen is decreased with ionophore inclusion (Van Nevel and DeMeyer, 1977; Dellinger and Ferry, 1984), subsequently reducing hydrogen production. Reducing hydrogen production reduces the efficiency and extent of CH₄ formation by methanogens (Czerkawski et al., 1972).

Studies reporting CH₄ reductions from ionophore inclusion in cattle diets vary both in magnitude and length of response (Sauer et al., 1998; Guan et al., 2006; Odongo et al., 2006). Ionophore responses are dependent on diet composition as well as type and amount of ionophore administered. *In vivo* studies have found a reduction in CH₄ production up to 30.7% (Table A1) while *in vitro* studies report inhibition ranging from 0 to 76% (as shown in table 4 of van Nevel and DeMeyer, 1996). Different methods utilized to quantify CH₄ production may explain some of the differences in findings (Johnson and Johnson, 1995). O'Kelly and Spiers (1992a) reported a 10.5% reduction in daily feed intake due to monensin feeding and a 25.6% reduction in CH₄ production. Odongo et al. (2006), however, reported only a 6.5% reduction in CH₄ production resulting from monensin inclusion. On average, short-term (\leq three weeks) ionophore inclusion decreases *in vivo* methanogenesis by 14.3%.

Although it is evident that ionophore inclusion decreases CH₄ emissions in the short term, long-term (\geq three weeks) effects of ionophore treatment on CH₄ mitigation are inconsistent. Monensin reduced CH₄ production by 6.5% and sustained this reduction for six months in lactating dairy cows (Odongo et al., 2006). However, Rumpler et al.

(1986) reported CH₄ production returned to control values by d 12 of monensin supplementation in beef steers. Additionally, CH₄ production in a study by Guan et al. (2006) returned to baseline level by the third week of monensin inclusion when cattle were fed a high forage diet and by the sixth week of monensin inclusion when fed a high concentrate diet. While these observations are contradictory, in general results indicate that ionophore-based inhibition of methanogenesis declines in the long-term; on average, long term ionophore inclusion decreases *in vivo* methanogenesis 8.2%. For this reason, rotation systems are often implemented to alleviate the long-term decline in effectiveness of feeding a single ionophore.

Volatile Fatty Acid (VFA) impacts. Hydrogen-producing bacteria are more apt to produce acetate and butyrate than propionate and succinate (Russell and Houlihan, 2003); thus, their inhibition alters the proportion of VFA present in the rumen. Propionate is the most reduced VFA and is gluconeogenic in the animal, therefore it is the VFA most efficiently utilized (Yokoyama and Johnson, 1988). Molar proportions of ruminal propionic acid increased from 31.9 to 41.0 and 43.5% for 100 and 500 mg monensin · head⁻¹ · day⁻¹ respectively, and maintained this increase throughout a 148 d experiment (Richardson et al., 1976). In a meta-analysis of studies by Ellis et al. (2012) monensin inclusion consistently resulted in a decreased acetate:propionate ratio (increased molar proportion of propionate in the ruminal fluid) which appeared to be related to decreased CH₄ production per unit feed (Ellis et al., 2012). A decreased acetate:propionate ratio is associated with reduced CH₄ production because reducing equivalents are being disposed of in an alternative electron sink, in this case propionate

(Yan et al., 2010; Ellis et al., 2012). Despite changes in molar proportions of acetate and propionate resulting from ionophore inclusion, total VFA production remains unchanged (Richardson et al., 1976; Guan et al., 2006). Therefore, ionophore feeding results in a shift of reducing equivalents to increase propionate and reduce CH₄ production, leading to greater energy retention by the animal from feed.

Effect on ammonia-producing bacteria

Protein fermentation. Ruminal protein fermentation is generally considered wasteful and as such ruminant nutritionists seek methods for reduction. Amongst the many benefits of including ionophores in ruminant diets is their impact on the ruminal nitrogen (N) pool, particularly concerning fermentation of protein.

Protein is categorized as either ruminally degradable or undegradable; degradable protein includes both true protein as well as non-protein N. Ruminal bacteria release a variety of proteases, peptidases, and deaminases into the ruminal environment that are active in the fluid (Wu, 2013). Several key factors, including protein structure (primary, secondary, tertiary, and quaternary), pH and predominant microbial species, determine the susceptibility of protein to degradation by these enzymes (Bach et al., 2005). True protein is degraded to oligopeptides, peptides, and free amino acids. These products are then further broken down, deaminated into NH₃-N, and fermented to produce VFA and energy. The carbon skeletons, NH₃, and energy can be used by microorganisms to synthesize microbial protein.

Ruminal ammonia production and N recycling. Ruminal NH₃ production is an important factor to consider in understanding the effect of ionophores on ruminant N

metabolism. Deaminases are the principle enzyme in the rumen responsible for the irreversible loss of amine groups from dietary amino acids as NH_3 (Wu, 2013). A minimum concentration of 50 mg $\text{NH}_3\text{-N/L}$ will support maximum growth rates of rumen bacteria (Satter and Slyter, 1974). Ruminant NH_3 is used in the synthesis of 50-80% of microbial N. If dietary degradable protein exceeds ruminant requirements, rate of protein fermentation may exceed the rate of ruminant NH_3 utilization and assimilation by microorganisms resulting in the accumulation of NH_3 in the rumen. Ammonia in ruminant fluid can be utilized for biosynthetic processes by rumen epithelial cells (Wu, 2013). Ammonia not utilized for amino acid or polypeptide synthesis in microorganisms is absorbed across the rumen wall, enters the portal blood, and is converted to urea in the liver (Attwood et al., 1998). Some urea from the blood is recycled back to the rumen by diffusion across the rumen epithelium or through saliva (Kennedy and Milligan, 1980). Urea is hydrolyzed by microbial urease in the rumen to form NH_3 and CO_2 . This recycling of urea-N aids in the conservation of NH_3 for biosynthetic processes in the rumen. Some of the urea, however, is lost in urine, resulting in decreased whole animal N retention.

Obligate amino acid-utilizing bacteria. Although much of the protein that enters the rumen is fermented, Bladen et al. (1961) noted that many pure cultures of ruminant bacteria were unable to produce NH_3 . Traditionally, ruminant bacteria were isolated in media containing carbohydrates and either NH_3 or low concentrations of protein hydrolysate as a N source (Chalupa, 1977). El-Shazly (1952) reported that the potential rate of ruminant NH_3 production from hydrolyzed protein by ruminant microorganisms

correlated with the amount of protein fed to the animal. Strains of *Bacteroides* (now *Prevotella*) *ruminicola*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Eubacterium ruminantium*, and *Butyrivibrio fibrosolvens* were found to produce some NH₃, but even with a 96 hr incubation period, less than 25% of the casein hydrolysate was converted to NH₃ (Bladen et al., 1961).

Bladen et al. (1961) determined that most strains produced little NH₃ and that, based on bacterial activities and numbers in the rumen, *Bacteroides* (now *Prevotella*) *ruminicola* was the most important NH₃-producing bacterium in the rumen of mature cattle. It was later determined that this species specific activity of NH₃ production was less than the specific activity of mixed cultures and could not account for all of the NH₃ accumulation *in vivo* (Russell, 1983). Hungate (1966) encountered rumen bacteria capable of digesting casein without carbohydrate in the media, however, he did not isolate them.

In the 1980s, enrichment cultures including high concentrations of protein hydrolysate yielded three ruminal bacterial species with specific activities of NH₃ production 18 to 39 times higher than previously identified species (Russell et al., 1988). These bacteria, did not utilize carbohydrates, rather, they utilized amino acids as their sole carbon and energy source, and are known as “obligate amino acid-fermenting” or “hyperammonia-producing bacteria” (Russell et al., 1988). Characterized by high specific activities of NH₃ production, these bacteria were later determined, by 16S rRNA sequence analyses, to be *Peptostreptococcus anaerobius*, *Clostridium stricklandii*, and a new species designated *Clostridium aminophilum* (Paster et al., 1993). Yang and Russell

(1993) used most-probable number estimates to determine that these obligate amino acid-utilizing bacteria accounted for less than 10% of the total ruminal bacteria count; but calculated that these species were capable of deaminating over 25% of protein in feeds (Krause and Russell, 1996). Although the extent of their role in the rumen remains unclear, it is critical to recognize their sensitivity to ionophores.

Much of the decrease in ruminal NH₃ production resulting from ionophores can be directly attributed to a 10-fold decrease in obligate amino acid-utilizing bacteria populations caused by ionophores (Krause and Russell, 1996). In early studies, addition of monensin to the diet tended to reduce ruminal NH₃ concentration *in vivo* (Dinius et al., 1976) and *in vitro* (Russell et al., 1988; Chen and Russell, 1989). Lower ruminal NH₃ concentrations may potentially reduce absorption of NH₃ into the blood resulting in a subsequent reduction in urinary N excretion. Mixed results, however are reported by Rogers et al. (1997) in regards to the protein sparing effect, but total tract N digestibility was consistently increased by monensin feeding (Muntifering et al., 1980; Faulkner et al., 1985). Additionally, *in vitro* studies have demonstrated a reduction in protein degradation, NH₃ accumulation, and microbial protein both in pure culture (Russell et al., 1988; Chen and Russell, 1989) and mixed culture (Van Nevel and Demeyer, 1977; Russell and Martin, 1984).

In addition to inhibition of obligate amino acid-utilizing bacteria, ionophores also inhibit the degradation of protein hydrolysate intermediates (e.g. poly and oligo-peptides) as well as protein (Russell and Martin, 1984). Chen and Russell (1991) suggested that monensin inhibits deamination more than proteolysis based on the

accumulation of α -amino-N and peptides. Significant quantities of peptide N could not be degraded by ruminal microorganisms and monensin feeding increased peptide flow from the rumen (Chen and Russell, 1991). Similarly, van Nevel and Demeyer (1977) reported that monensin reduced protein degradation and NH_3 production while resulting in the accumulation of α -amino N. These data suggest the potential for a greater proportion of dietary true protein to escape the rumen (protein sparing) when monensin is added to the diet (Muntifering et al., 1980). Overall, monensin treatment results in a decline in deaminating activity of the rumen population, resulting in increased N retention.

Protein sparing effect of monensin. Ionophore supplementation can have a profound impact on ruminal N retention (Russell and Strobel, 1989). Because of the sensitivity of obligate amino acid utilizing bacteria to monensin, ruminal amino acid degradation and resulting NH_3 accumulation are decreased by 50% (Dinius et al., 1976). The overall beneficial effect of ionophores on ruminal amino acid degradation and the resultant decrease in NH_3 production is termed “protein sparing” (Russell and Strobel, 1989). Because monensin increases dry matter digestibility and shifts ruminal fermentation toward increased propionate production, amino acids are spared from deamination allowing for their availability to the animal in the post-ruminal gastro-intestinal tract. Ørskov et al. (1979) demonstrated the protein sparing effect by increasing N retention when high levels of propionate were infused into the rumen of sheep. Digestibility of dietary protein is generally greater than bacterial protein (Van Soest, 1994) and the

greater amount of dietary protein escaping the rumen compared with bacterial protein provides for the metabolic protein sparing effect.

Effect on ruminal protozoa

Association of methanogenic archaea with ciliate protozoa. As integral components of the rumen ecosystem, methanogenic archaea utilize reducing equivalents produced by hydrogen-producing bacteria, anaerobic fungi, and ciliate protozoa (Tokura et al., 1999). Defaunation of protozoa from the rumen has been associated with a 30-45 % reduction in CH₄ (Jouany, 1991; Ushida et al., 1997), indicating a close relationship between ruminal methanogens and ciliate protozoa. Defaunation also results in decreased protein degradation and concentrations of peptides and amino acids in the rumen (Ivan et al., 1991).

The association of methanogenic archaea with rumen ciliates is a common feature of anaerobic systems (Vogels et al., 1980). Methanogens are often observed on the outer surface of protozoa due to their epifluorescence; methanogenic archaea possess fluorescing coenzymes F₃₅₀ and F₄₂₀ (Doddema and Vogels, 1978). The attachment of methanogenic archaea to ciliate protozoa is of value to both organisms since it provides the capacity for interspecies hydrogen transfer (Wolin, 1974). Ciliate protozoa depend on a hydrogen evolving fermentation that provides substrate for methanogens. Methanogens attached to protozoa derive H₂ directly from specialized organs called hydrogenosomes (Hungate, 1966). Protozoa benefit from hydrogen removal because of its inhibitory effect on their metabolism if not removed (Sharp et al., 1998).

Although the association of methanogens with the outer surface of ciliates was discovered long ago, more recent research has revealed methanogens living inside rumen ciliates. Early evidence that intracellular bacteria might be methanogens came from observations of their UV-induced autofluorescence. The first documented case of methanogens living inside rumen ciliates was provided by Finlay et al. (1994) when intracellular bacteria free in the cytoplasm of the most abundant ciliate species, *Dasytricha ruminantium* and *Entodinium* spp. were observed. It was confirmed by electron microscopy that the bacteria were enclosed in membrane-bounded vacuoles. These bacteria were readily detected because of their F₄₂₀ autofluorescence. It was suggested that all of the intracellular bacteria were likely methanogens, accounting for approximately 1-2 % of host ciliate volume.

Effect of ionophores on rumen protozoa. Although ruminal protozoa are not essential for feed digestion, they can account for half of the microbial mass in the rumen. Bladen (1961) suggests ruminal protozoa are of considerable significance in NH₃ production from amino acids and differences in numbers or species of protozoa might be involved. The capacity of protozoa to produce NH₃ is less than that of bacteria (Hino and Russell, 1987) but, because they are involved in the recycling of bacterial protein, defaunation tends to decrease ruminal NH₃ concentrations (Eadie and Gill, 1971). Rumen protozoa are sensitive to monensin *in vitro* (Hino, 1981; Dennis et al., 1986) and *in vivo* (Dennis et al., 1986; Habib and Leng, 1986), however, the degree of sensitivity may vary with species. Hino (1981) suggests that protozoa modify their fermentation patterns in the presence of ionophore; implying the effect on protozoa may be diet dependent.

Effect on lactic acid-producing bacteria

Lactate is a stronger acid (pKa 3.86; National Center for Biotechnology Information, 2014) than the three major volatile fatty acids (pKa: acetate 4.74, propionate 4.87, butyrate 4.82; National Center for Biotechnology Information, 2014), thus accumulation decreases ruminal pH leading to ruminal acidosis. Ruminal acidosis has been associated with reduced feed intake, lowered feed efficiency (Callaway et al., 2003), ulceration, founder, and in severe cases, death (Russell and Strobel, 1989). Most lactate-producing rumen bacteria (*Butyrivibrio fibrosolvens*, *Eubacterium cellulosolvens*, *E. ruminantium*, *Lachnospira multiparus*, *Lactobacillus ruminis*, *L. vitulinus*, *Rumiococcus albus*, *R. flavofaciens*, *Streptococcus bovis*) are inhibited by ionophores while succinate producers (*Bacteroides*, *Selenomonas*, *Succinimonas*, *Succinivibrio*) and lactate fermenters (*Anaerovibrio*, *Megasphaera*, *Selenomonas*) are not (Dennis et al., 1981). The inhibition of major lactate-producing bacteria suggests that ionophores may be used for prevention of lactic acidosis in ruminant animals.

Effect on dietary intake and gain

Considering the multitude of effects that ionophores have on the ruminant animal, it is important to consider overall performance effects, such as changes in dry matter intake and average daily gain. These effects are of particular importance to producers as they directly drive profitability. Ionophores increase pH, creating a more ideal environment for rumen microbial populations increasing fermentation and utilization of nutrients. Accordingly, ionophores are reported to decrease intake while average daily gain remains unchanged (Byers, 1980; Delfino et al., 1988; O'Kelly and Spiers, 1992a).

Byers (1980) reported similar rates of live weight gain for *ad libitum* fed cattle with and without monensin (920 vs 923 g/day, respectively), but dry matter intake was significantly reduced (5.91%) with monensin. Monensin improved feed conversion (5.84%) but terminal body composition and total energy retained were not different between the treatments. In another study, O'Kelly and Spiers (1992a) reported a 10.5% reduction in daily feed intake due to monensin and a 25.6% reduction in CH₄ production. Similarly, Delfino et al. (1988), reported heifers fed monensin or lasalocid at 54 mg/kg required less ($P<0.05$) DM/kg gain than control heifers in an initial 28 d period. Overall, heifers receiving diets containing lasalocid at 36 or 54 mg/kg required 6.2 kg DM/kg gain, whereas monensin-fed and control heifers required 6.5 and 6.9 kg DM/kg gain ($P<0.01$), respectively (Delfino et al., 1988). Changes in intake and gain are a result of the altered energy partitioning induced by ionophores.

Effect on energy partitioning

Byers (1980) suggests that the maintenance energy requirement of an animal may be reduced when an ionophore is fed by increasing the metabolizable energy value of the diet. It is necessary therefore, to quantify effects of ionophores on energy partitioning and performance to determine the mechanisms by which responses are mediated.

Digestible energy. Fecal energy losses are an important consideration in energy metabolism because of the inherent association with digestible energy, but the effects of ionophores vary. Thornton and Owens (1981) reported that fecal energy loss as a percent of gross energy intake was not altered significantly at any of three roughage levels by the addition of monensin. Fecal energy losses reported by Wedegaertner and Johnson

(1983), however, were significantly different ($P<0.01$); $88.7 \text{ kcal} \cdot \text{metabolic body size}^{-1} \cdot \text{d}^{-1}$ fecal energy loss for monensin fed steers compared with $98.6 \text{ kcal} \cdot \text{MBS}^{-1} \cdot \text{d}^{-1}$ for control fed steers. Percentage of digestible energy increased from 71.8% for control to 74.8% with monensin inclusion (Wedegaertner and Johnson, 1983). The increase in digestible energy was largely accounted for by an increase in neutral detergent fiber (NDF) digestion ($P<0.01$) from 50.5% for control to 57.5% for monensin fed steers. Similarly, Delfino et al. (1988) reported a 6.3% increase in digestible energy resulting from lasalocid inclusion. With this data, it can be postulated that ionophores increase a ruminant animal's ability to digest NDF by decreasing intake, thereby increasing retention time allowing for greater extent of digestion.

Metabolizable energy. Energy lost in the form of urine and CH_4 directly impact metabolizable energy, ionophores do not significantly alter urinary energy lost as a percent of GE intake (Thornton and Owens, 1981; Wedegaertner and Johnson, 1983; Delfino et al., 1988). Methane energy loss, however, substantially decreases as a result of ionophore inclusion. Enteric CH_4 production is associated with a 2 – 15% loss of gross energy intake for the ruminant animal (Johnson and Johnson, 1995; Moss et al., 2000; Van Nevel and Demeyer, 1996).

Studies reporting CH_4 reductions from ionophore supplementation in cattle vary both in magnitude and length of response (Sauer et al., 1998; Guan et al., 2006; Odongo et al., 2006). In an effort to prolong the beneficial effects of ionophore inclusion, rotation programs have been investigated and will be reviewed in a subsequent section. However, because ionophores decrease CH_4 production for a period of time, CH_4 is reduced per

unit product (meat or milk; Tedeschi et al., 2003) therefore reducing CH₄ emissions from ruminant livestock to the atmosphere and improving overall feed efficiency. An average 7.5% of digestible energy was lost as CH₄ for control steers and 7.1% for steers fed lasalocid (Delfino et al., 1988). Lasalocid improved metabolizable energy density of the diet by 8, 8, and 5% for feeding levels of 21, 44, and 67 g DM/kg MBW (Delfino et al., 1988). Improved feed conversion of the diet was attributed to the increase in metabolizable energy density. Wedegaertner and Johnson (1983) reported a 26% reduction in CH₄ production for steers fed a cracked corn-silage diet. In total, monensin increased metabolizable energy from 63.3 to 66.8% of gross energy intake (Wedegaertner and Johnson, 1983). The increase in metabolizable energy was the sum of monensin effects on fecal, urinary, and CH₄ losses. Similarly, Thornton and Owens (1981) reported monensin decreased CH₄ production by 16% at low (14% roughage; 12% ADF) and medium (46.2% roughage; 27% ADF) roughage levels, and by 24% at a high (65.7% roughage; 40% ADF) roughage level. Addition of monensin did not significantly affect metabolizable energy at any roughage level; however, when averaged across roughage levels, metabolizable energy was increased 5.2% by monensin (Thornton and Owens, 1981). The increase is explained partially by the decreased CH₄ energy loss and partially by a tendency for monensin to decrease fecal energy loss. On average, van Nevel and DeMeyer (1996) indicated that short-term ionophore inclusion caused an 18% decrease in *in vivo* methanogenesis for the studies reported.

Net energy. Although heat production is not always altered (Thornton and Owens, 1981), it has been reported to increase (Wedegaertner and Johnson, 1983) with

ionophore inclusion. At equal gross energy intakes, increases in heat production reflected the increased metabolizable energy available when monensin was included in the diet (Wedegaertner and Johnson, 1983); thus it was concluded that monensin does not affect the heat increment of metabolizable energy used for maintenance or gain, or the metabolizable energy requirement for maintenance. As a result, both NE_m (mcal/kg) and NE_g (mcal/kg) were increased by approximately 7% by monensin (Wedegaertner and Johnson, 1983). Similarly, Joyner et al. (1979) reported control fed lambs produced 1,987 kcal heat/d while monensin fed lambs produced 2,093 kcal heat/d ($P < 0.05$) which was proportional to increased metabolizable energy density of the diet. Delfino et al. (1988) also reported average heat productions of steers to be increased ($P < 0.05$) by 7% with lasalocid inclusion. Although NE_m and NE_g content of the diet were not influenced with lasalocid, there was a 10-21% difference between dietary treatments for NE_m (Delfino et al., 1988).

Retained energy. Wedegaertner and Johnson (1983) report that an increase in metabolizable energy resulted in a 19.9% increase in retained energy when monensin was included in the diet. Similarly, Byers (1980) reports energy retention of control and monensin fed cattle, with equal dry matter intake, to increase 6.6%. In contrast, Delfino et al. (1988) reported no difference in retained energy between lasalocid and control fed steers. Conflicting data exist regarding the effect of ionophores on retained energy; however, data indicate the possibility of a difference in effect being due to type of ionophore fed.

Data indicate that the majority of improved energy partitioning occurs through the effect of monensin on reduced fecal and CH₄ energy losses. Although the improvement in fecal loss is greater, decreasing CH₄ loss has the additional advantage of reducing the impact of ruminant animals on global greenhouse gas emissions.

Mechanisms of ionophore resistance and potential impact on human health

Potential impact on human health

Antibiotic effectiveness has been reduced in many cases because bacteria freely transfer genes encoding for resistance factors between species (Salyers and Shoemaker 2006; Salyers et al., 2007). Although humans are not prescribed ionophores for treatment of bacterial infection, the concern exists that ionophores pose a threat to public health in regard to antibiotic resistance. It is important when examining antimicrobial resistance in a bacterial species to determine if resistance results from selection of intrinsic resistance or emergence of novel resistant populations (Callaway et al., 2003). Some ruminal microorganisms are intrinsically resistant (insensitive) to ionophores even when ionophores are not fed; other species are sensitive to ionophores, but can become or acquire a reduced sensitivity (resistance; Dawson and Boling, 1983; Newbold et al., 1988; Callaway et al., 1999).

Mechanism of resistance

Bergen and Bates (1984) suggested that ionophore resistance was related to the membrane-bound enzyme fumarate reductase, a proton-translocating enzyme, because ruminal bacteria that produce succinate and propionate were resistant and hypothesized that this enzyme might counteract ionophore-dependent ion flux. Morehead and Dawson

(1992) noted the appearance of more fumarate reductase activity in monensin-resistant *Prevotella ruminicola* strains than monensin-sensitive strains. This hypothesis was contradicted by Chen and Wolin (1979) who found *Ruminococcus flavefaciens*, a fumarate reductase-containing species that produces large amounts of succinate, to be highly sensitive to monensin. Sensitivity of ruminal microorganisms to ionophores has been suggested to be relatively stable because the pattern of resistance results from differences in cell membrane structures (Russell and Strobel, 1989). Dawson and Boling (1983) monitored monensin-sensitivity in calves. They found nearly 60% of bacterial isolates were monensin-sensitive prior to treatment with only a marginal increase as a result of subsequent monensin administration.

Although gram-positive bacterial species are generally ionophore sensitive, some gram positive bacteria adapt following repeated exposure to the ionophore (Dennis et al., 1981; Dawson and Boling, 1983; Newbold et al., 1988; Callaway et al., 1999). Callaway et al. (1999) found some gram-positive bacteria to be sensitive to monensin initially, but, following repeated exposure, these bacteria were able to significantly increase their resistance. Ionophore resistance appeared to result from an increase in extracellular polysaccharides (glycocalyx). Extracellular polysaccharides may play a key role in ionophore resistance of ruminal bacteria (Russell and Houlihan, 2003) because they prevent binding of the ionophore to the cell wall (Rychlik and Russell, 2002). Upon withdrawal of the ionophore, resistant bacteria did not persist in the population, indicating changes were a result of selection of naturally resistant subpopulations as opposed to emergence of novel/acquired traits (Callaway et al., 2003).

Ionophore resistance of bacterial populations have been monitored by measuring the amount of ionophore required to catalyze K^+ depletion from bacterial cells due to the K^+ efflux from ionophore-sensitive cells (Callaway et al., 1999; Lana and Russell, 1996). Rapid K^+ depletion occurred when mixed ruminal bacteria were collected from ionophore treated cattle consuming hay; rumen fluid collected during the 4 days prior to treatment and 1,2,3,4,5,8,12,15, and 18 d after treatment (Lana and Russell, 1996). Resistance of the mixed ruminal bacterial population was significantly greater within 3 d of initiation of daily ionophore supplementation (Lana and Russell, 1996).

There is little rationale for common mechanisms of resistance between traditional antibiotics and ionophores (other than glycocalyx formation) because ionophores utilize a different mode of action than most therapeutic antibiotics (Russell and Houlihan, 2003). Due to the intrinsic physiological mechanisms of ionophore resistance, there is little evidence that ionophore resistance can be spread from one bacterium to another as with therapeutic antibiotics. After many years (> 35 years in the U.S) of widespread use, highly ionophore-resistant isolates have rarely been isolated (Aarestrup et al., 1998, 2000), and ionophores continue to improve efficiency of ruminal fermentation. Russell and Houlihan (2003) concluded, based on collective observations, that use of ionophores in animal diets is not likely to significantly impact transfer of antibiotic resistance from animals to man.

Ionophore rotation programs

Ionophore rotation programs are a suggested method to alleviate the long-term loss of effectiveness of a single ionophore and to maximize the reduction of CH_4 production.

Potential benefits of rotation have been hypothesized to result from reduced adaptation to a specific ionophore (Morris et al., 1990) and altered site and extent of digestion (Galyean and Hubbert, 1989). Johnson et al. (1988) found a daily rotation of lasalocid and monensin plus tylosin (an antimicrobial) improved daily gain and feed efficiency in feedlot cattle compared with feeding lasalocid or monensin plus tylosin continuously. Branine et al. (1989) also suggested that feeding a daily rotation of monensin and lasalocid enhanced feed efficiency and gain more than continuously feeding either ionophore alone. However, Morris et al. (1990) found no differences in the effect of daily rotation of lasalocid and monensin plus tylosin, concluding that a daily rotation scheme was too frequent to overcome any possible adaptation to ionophores. Guan et al. (2006) investigated a rotation of monensin and lasalocid every two weeks, but found that it did not extend the duration of reduced enteric CH₄ emission. Thus, the extent and mode of action of the synergistic benefit of ionophore rotation remains unclear.

Conclusions

The livestock industry has come to rely on the use of ionophores in ruminant rations because of their impact on reducing CH₄ production and energy loss thereby providing an economic benefit to the producer. Ionophore action in the rumen is primarily due to sensitivity of targeted bacterial species, ion selectivity, and concentration gradient of ions which can be translocated by the ionophore. Cell membrane physiology appears to be the determining factor in sensitivity. Coincidentally, sensitive organisms generally produce more NH₃, hydrogen, and lactate than do resistant species. Reduction of hydrogen-, NH₃-, and lactic-acid producing bacteria are responsible for improved feed

efficiency related to ionophore inclusion. These reductions decrease the acetate:propionate ratio, decreasing production of CH₄; increase protein availability; increase pH, decreasing the occurrence of acidosis; and increase the percent of feed digested. Additionally, CH₄ mitigation contributes to a reduction in the contribution of ruminant animals to concentration of global greenhouse gases. Thus, even in the case of acquired resistance, ionophores are effective on a global scale for improving CH₄ production per unit product.

Ionophores use a different mode of action than most therapeutic antibiotics, thus, their use is not likely to significantly impact transfer of antibiotic resistance from animals to man. In an effort to alleviate the long-term loss of effectiveness of a single ionophore, ionophore rotation programs have been implemented, but the extent and mode of action of the synergistic benefit remains unclear.

Although a variety of feed additives have been developed through the years, ionophores have continued to improve ruminant animal production efficiency and mitigate enteric CH₄ production since their 1975 approval for use in the United States. Thus, the use of ionophores is consistent with a more sustainable, economically and energy efficient, environmentally-friendly, cattle production system.

Effect of protein supplementation on protozoa, methanogen, and obligate amino acid-utilizing bacterial populations in the rumen

Continued use of ruminant animals for meat, milk and wool production is justified by their ability to ferment cellulolytic materials unusable by humans and their ability to

utilize non protein nitrogen (NPN) and low-quality sources of protein to supply the host animal with microbial protein. Cellulose is the most abundant organic molecule on earth and ruminants are able to ferment cellulolytic materials, reducing their direct competition with humans for food calories. It is common in beef cattle production systems to encounter scenarios where nutrient quality is low or sufficient quantities of nutrients are unavailable. Under these conditions, supplementation is used to more closely align nutrient supply and animal requirements. Ideally, diet formulation should allow a supplement to complement the available forage such that nutrient requirements are met and the proportion of nutrients derived from forage is optimized. To achieve this goal, it is important to understand the impact of specific constituents of a supplement on forage utilization.

Protein supplementation for grazing or forage-fed cattle

Ruminant protein requirements are met via dietary protein and microbes grown in the rumen and digested in the small intestine. Microbial protein has relatively good amino acid balance (Clark et al., 1992) and digestibility (NRC, 1996). It represents the greatest source of protein for ruminant animals (Firkins, 1996), providing 2/3 of their requirement (Iskenderov and Mamedova, 2013). Ruminant nutritionists seek to increase production efficiency by maximizing flow of microbial protein to the small intestine. Level of microbial protein synthesis is directly tied to the amount of carbohydrate digested in the rumen (NRC, 1996). When ruminant animals receive high quality forage (CP \geq 7%), they are able to synthesize microbial protein at higher levels than when they receive LQF. Cattle feeding systems commonly depend on low-quality forage (LQF; CP

< 7%) which consists largely of fibrous (e.g. cellulosic) materials, and relatively low digestibilities. Although LQF provides significant quantities of carbohydrate, their use is dependent on cellulolytic activity of ruminal microorganisms. Ruminal cellulolytic bacteria require branched chain fatty acids for growth (Dehority et al., 1967); these acids are mostly formed by degradation and deamination of dietary branched-chain amino acids (El Shazly, 1952), thus growth and fermentation is dependent, specifically, upon ruminally available N (Cochran et al., 1998). An inadequate supply of ruminal N reduces fermentation, digesta outflow, and ultimately intake (Maeng et al., 1976). Thus, a knowledge of the nitrogenous compounds utilized for microbial growth is essential for understanding and improving ruminal cellulose digestion and microbial protein synthesis.

Feedstuff protein is partitioned into two fractions, ruminally degradable intake protein (DIP) and undegradable intake protein (UIP). Degradable intake protein is degraded by rumen microorganisms and is used to address the nutrient requirements of ruminal bacteria (Allison, 1969). Consequently, by maintaining ruminal fermentation and promoting microbial growth, DIP increases cellulolytic activity and microbial protein synthesis thereby improving nutrient utilization from forages deficient in DIP. Rumen microorganisms are incapable of degrading UIP and it is transported to the small intestine for enzymatic digestion and potential absorption (NRC, 1996). Accordingly, UIP cannot directly contribute to microbial protein production (Cochran et al., 1998). Undegradable intake protein that is absorbed and not used for maintenance or production can be deaminated by the liver. The resultant $\text{NH}_3\text{-N}$ can then be used to synthesize urea,

which can enter the rumen by diffusion across the rumen wall and from saliva, for use as a source of ruminally available N. In this manner, UIP can indirectly contribute to microbial protein production.

Low-quality forage diets are generally more effectively utilized when supplemented with DIP than UIP (Köster et al., 1996; Cochran et al., 1998). The quantity of DIP needed to optimize microbial growth may, under some conditions, be as high as 14 to 15% of dietary DM (Hoover and Stokes, 1991). Ruminant animals consuming LQF and supplemented with DIP, exhibit improved forage intake, digestion, and overall animal performance (DelCurto et al., 1990; Köster et al., 1996; Mathis et al., 1999; Bandyk et al., 2001).

Effects of protein supplementation on utilization of LQF at the animal level are well documented; however, effects on specific rumen microbial populations are limited. Furthermore, characterizing the differences in microbial populations in Bt and Bi subspecies of cattle are non-existent. Quantity and diversity of bacteria in the rumen influence microbial protein synthesis (NRC, 1996) and therefore utilization of LQF. Understanding these population differences will enable a greater understanding of the host-microbe relationship and its impact on animal performance. Of particular interest are methanogenic archaea, ciliate protozoa, and obligate amino acid-utilizing bacteria, thus, literature concerning these particular microbial populations will be reviewed.

Methanogenic archaea

Prior to the 1970's, all bacteria were classified into a single prokaryotic kingdom. By analyzing rRNA sequences, Woese and Fox (1977), recognized that variable regions of

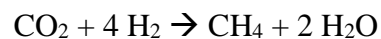
rRNA could be used to trace genetic linkages. Using this technique, they determined that methanogens, which lack a typical peptidoglycan and possess membrane lipids composed of ether linkages rather than ester linkages, are no more related to other prokaryotes than they are to eukaryotes (Woese and Fox, 1977). This discovery led to the creation of a new domain, Archaea, which includes organisms known to have novel physiological features and that are only distantly related to other bacteria (Woese and Fox, 1977). Methanogens, a specialized group of Archaea, utilize fermentation products (e.g. formate and H₂) to produce CH₄, were the first group of organisms to be included in the new domain.

Fermentation products from pure cultures of rumen bacteria grown *in vitro* include: carbon dioxide, CH₄, hydrogen, formate, acetate, propionate, butyrate, lactate, succinate, and ethanol. Carbon dioxide, CH₄, acetate, propionate, and butyrate are considered end products of fermentation, which is supported by their presence in the rumen. Hydrogen, ethanol, formate, lactate, and succinate, in contrast, do not ordinarily accumulate in the rumen; however, on occasion, large quantities of lactate are observed (Hungate, 1966). It is assumed that the same products formed in pure culture are produced by bacteria in the rumen and the absence of hydrogen, ethanol, formate, lactate, and succinate suggests their roles as intermediates, which are converted into the aforementioned final products.

Syntrophic H₂ transfer was first hypothesized nearly 50 years ago (Hungate, 1966) and has a profound impact on the balance of fermentation products in the rumen. Cross-feeding benefits methanogens, bacteria, protozoa and fungi by allowing them to obtain more energy for growth (Krause et al., 2013). Syntrophic interspecies H₂ transfer is

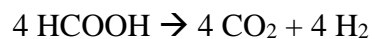
considered to be a critical ecological component in nearly all methanogenic ecosystems (Thiele and Zeikus, 1988).

As integral components of the rumen ecosystem, methanogenic archaea utilize reducing equivalents produced by hydrogen-producing bacteria, anaerobic fungi, and ciliate protozoa (Tokura et al., 1999). Methane and acetic acid are the two possible products of anaerobic hydrogen and carbon dioxide conversion in the rumen.



Few bacteria able to convert hydrogen and carbon dioxide into acetic acid have been found in the rumen indicating the primary fate of hydrogen is CH₄ (Hungate, 1966).

Formate produced in the rumen is rapidly converted and can be recovered as CH₄ and carbon dioxide (Hungate, 1966). The initial conversion involves the production of carbon dioxide and hydrogen.



Subsequently, the carbon dioxide and hydrogen products are converted to CH₄.

Although CH₄ emission is an energy loss for the animal and increases the contribution of ruminant systems to global greenhouse gas emission, the removal and use of H₂ by rumen methanogens is critical for optimal microbial fermentation. An accumulation of reducing equivalents (primarily NADH₂) in the rumen slows the rate of fermentation and efficiency unless H₂ is redirected into alternate electron sinks.

Effect of protein supplementation on methanogenic archaea

Inadequate ruminally available-N limits growth of cellulolytic bacteria, decreases fermentation rate and passage rate, and reduces intake. Fermentation of fiber yields a greater proportion of acetic acid than propionic (Johnson and Johnson, 1995), and is more methanogenic than fermentation of soluble carbohydrates (Moe and Tyrrell, 1979). It can be presumed then, that consumption of LQF increases the concentration of methanogenic archaea in the rumen.

Degradable intake protein supplements allow growth of cellulolytic bacteria resulting in more effective forage utilization, increased intake and digestion. Methane losses are increased in proportion to the improvement in digestibility by ammoniation (Birkelo et al., 1986). Overall CH₄ losses per unit product (meat, milk, or wool), however, would be decreased (Johnson and Johnson, 1995).

Rumen ciliate protozoa

Association of methanogenic archaea with ciliate protozoa

Association of methanogenic archaea with rumen ciliates is a common feature of anaerobic systems (Vogels et al., 1980). Defaunation of protozoa from the rumen has been reported to reduce CH₄ by 30-45% (Jouany, 1991; Ushida et al., 1997), indicating a close relationship between ruminal methanogens and ciliate protozoa.

Many protozoa exist with bacteria attached to their surface facilitating interspecies hydrogen transfer (Wolin, 1974). Ciliate protozoa depend on hydrogen evolving fermentation that provides substrate for methanogens. Protozoa benefit from hydrogen removal because of its inhibitory effect on their metabolism if not removed (Sharp, et al.,

1998). Methanogens attached to protozoa derive H₂ directly from specialized organs called hydrogenosomes (Hungate, 1966).

Although the association of methanogens with the outer surface of ciliates was discovered long ago (Vogels et al., 1980), more recent research (Finlay et al., 1994) has revealed methanogens living in the cytoplasm of common rumen ciliates, including *Dasytricha ruminantium* and *Entodinium* spp. (Finlay et al., 1994). This observation was confirmed by electron microscopy showing the bacteria enclosed in membrane-bounded vacuoles. Finlay et al., (1994) suggested that the intracellular bacteria were methanogens and accounted for approximately 1-2 % of host ciliate volume.

Effect of protein supplementation on protozoa

A ruminant diet deficient in ruminally available N (e.g. LQF) results in protozoal and bacterial lysis and degradation leading to intraruminal recycling of microbial N (Koenig et al., 2000). Protein supplementation increases protozoa concentration in the rumen by making N available.

Techniques for culture-based enumeration of methanogens are available; however, their association with protozoa make this difficult (Sharp et al., 1998). Increased protozoa populations would be expected to harbor a greater population of methanogenic archaea that would escape detection using current methods. Ultimately, this suggests an underestimate of number of methanogens in the rumen by most probable number methods. Accordingly, when an accurate estimation of methanogen populations is desired, the method of quantification must consider protozoal populations. More advanced molecular techniques may provide more accurate determinations.

Obligate amino acid-utilizing bacteria

Dietary protein is extensively degraded and fermented by ruminal microorganisms to NH_3 (Russell and Hespell, 1981). Ammonia is a source of N for bacterial growth, and is the preference of cellulolytic bacteria (Russell et al., 1992). Bladen et al. (1961) noted that many pure cultures of ruminal bacteria were unable to produce NH_3 . Traditionally, ruminal bacteria were isolated in media containing carbohydrates and either NH_3 or low concentrations of protein hydrolysate as a N source (Chalupa, 1977). El-Shazly (1952) reported that the potential rate of ruminal NH_3 production from hydrolyzed protein by ruminal microorganisms correlated with the amount of protein fed to the animal. Strains of *Bacteroides* (now *Prevotella*) *ruminicola*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Eubacterium ruminantium*, and *Butyrivibrio fibrosolvens* were found to produce some NH_3 , but even with a 96 h incubation period, less than 25% of the casein hydrolysate was converted to NH_3 (Bladen et al., 1961). They concluded that most strains produced little NH_3 and that, based on bacterial activities and numbers in the rumen, *Bacteroides ruminicola* (now *Prevotella bryantii*) was the most important NH_3 -producing bacterium in mature cattle. Subsequently, it was determined that specific activity of NH_3 production for this species was less than the specific activity of mixed cultures and could not account for all of the NH_3 accumulation *in vivo* (Russell, 1983).

Hungate (1966) observed rumen bacteria capable of fermenting casein without carbohydrate in the media, however, he did not isolate them. In the 1980s, enrichment cultures including high concentrations of protein hydrolysate yielded three ruminal bacterial species with specific activities of NH_3 production 18 to 39 times greater than

previously identified species (Russell et al., 1988). These bacteria, did not utilize carbohydrates, rather, they utilized amino acids as their sole carbon and energy source, and are known as “obligate amino acid-fermenting” or “hyperammonia-producing bacteria” (Russell et al., 1988). Characterized by high specific activities of NH_3 production, these bacteria were later determined, by 16S rRNA sequence analyses, to be *Peptostreptococcus anaerobius*, *Clostridium stricklandii*, and a new species designated *Clostridium aminophilum* (Paster et al., 1993). Yang and Russell (1993) used most-probable number estimates and determined that these bacteria accounted for less than 10% of the total ruminal bacteria count but were capable of deaminating over 25% of protein in feeds (Krause and Russell, 1996).

Effect of protein supplementation on obligate amino acid-utilizing bacteria

The effect of supplementing LQF with protein on concentration of obligate amino acid-utilizing bacteria is unclear. However, it can be presumed that increased N availability for rumen microbial growth would increase populations of obligate amino acid-utilizing bacteria. This would increase NH_3 production allowing for absorption across the rumen wall to the liver. In the liver, NH_3 will be converted to urea to be recycled back to the rumen or excreted in urine. Therefore, when LQF is fed, it can be hypothesized that protein supplementation will increase concentration of obligate amino acid-utilizing bacteria and rate of ruminal NH_3 production.

Subspecies differences in intake, digestion, and ruminal ammonia and methane production while consuming low-quality forage

Comparative studies of the digestive functions of *Bos indicus* (Bi) and *Bos taurus* (Bt) cattle have been conducted through the years in an effort to determine the specific mechanism that enables Bi cattle to better utilize nutritional components from LQF than Bt cattle (Howes et al., 1963).

Intake

Whilst a number of studies (Ledger, et al., 1970; Frisch and Vercoe, 1977; Hunter and Siebert, 1986; Habib et al., 2008) have observed Bt cattle to have greater intake than Bi cattle, this affect appears to be diet dependent. In a forage diet comprising high-quality German grass (*Echinochloa crusgalli*; 61 g CP/kg DM) and low-quality rice straw (48 g CP/kg DM) Habib et al. (2008) observed a 7.0% greater voluntary DM intake (1.99 vs 1.84 kg DM/d) for consumption of German grass by Bt ×Bi cross vs Bi steers. However, consumption of rice straw was not different between subspecies (1.35 vs 1.29 kg DM/d, Bt ×Bi vs Bi, respectively). Similarly, Hunter and Siebert (1986) observed that when Bi and Bt cattle consumed high-quality forage (lucerne or pasture hay and 2 kg lucerne; average 29.5 g N/kg OM), Bt consumption was 17.4% greater than Bi (26.3 vs 22.4 g DM/kg live weight, Bt and Bi, respectively). When low-quality forage (speargrass, pangolagrass and pasture hay; average 8.1 g N/kg OM) was offered, however, consumption by Bi and Bt cattle was similar (16.0 and 16.9 g DM/kg live weight). Furthermore, as N deficient diets are supplemented with protein, the magnitude of intake response is greater (35% vs 22%) in Bt than Bi (Hunter and Siebert, 1987).

Thus, as the quality of the diet improves, subspecies difference becomes progressively greater and variability between animals progressively smaller as a proportion of intake (Hunter and Siebert, 1986, 1987).

Digestion and fermentation

Although not significant, French (1940) observed the digestion coefficients for most parameters to favor Bi, and was the first to suggest greater fiber digestion in Bi than Bi × Bt consuming hay. Factors causing digestibility differences between subspecies were first investigated by Hungate et al. (1960) and Phillips et al. (1960). Hungate et al. (1960) and Phillips et al. (1960) suggested that Bi had faster rates of fermentation than Bt × Bt when low-quality forages were fed (17 and 18%, respectively); although neither were able to demonstrate significant differences, possibly due to the number of animals used. Phillips et al. (1960) indicated that Bi possess a shorter (8.7%) rumen retention time and greater (4.1%) dry matter digestibility when low-quality forages (6% CP) are fed. Rumen fermentation rate and retention time are negatively correlated (Phillips et al., 1960) with Bi having shorter retention time and higher fermentation rate than Bt × Bt.

Howes et al. (1963) observed higher coefficients of apparent digestion in Bi for all parameters except ether extract, however only the coefficients for crude protein were significantly ($P \leq 0.05$) different. Bi digested more protein over the lower range of dietary protein intakes, but the difference tended to disappear when the diet included a protein supplement. Similarly, Moore et al. (1975) observed that fermentation with rumen inocula from Bi was greater than Bt (71 vs 67.4% IVDMD) fed a low-protein (11.02% CP), low-energy (2425 kcal DE/kg) ration. Kennedy (1982) observed apparent

digestibilities of organic matter, cell wall constituents, and N to be lower for steers fed pasture hay than for steers fed alfalfa, however, no differences were observed between breeds. Numerically, Bi × Bt steers digested more digestible OM intake in the reticulo-rumen than did Bt steers (90 vs 83%). Habib et al. (2011) observed Bi to have greater OMD than Bt steers consuming low digestibility (58.0%) rice straw (61.2 vs 54.7%), but similar OMD when they consumed high digestibility (69.0%) German grass (68.5 vs 68.5%).

As with intake, forage quality appears to be a determining factor in the presence of a subspecies difference in digestion. The ability of Bi to ferment N-deficient diets more rapidly than Bt may be due to a greater quantity of urea being transferred to the digestive tract (Norton et al., 1979) and to the greater rumen protozoal population in Bi cattle (Hennessy et al., 1995). Greater protozoal density in Bi cattle would suggest a more rapid digestion of particulate proteins, which would reduce the outflow of proteins and perhaps also peptides and specific amino acids from dietary proteins (Hennessy et al. 1995).

Ruminal ammonia production

Hunter and Siebert (1985a) reported that greater *in situ* rate of digestion of Bi cattle was associated with greater rumen ammonia concentration when consuming Pangola grass (7.9 g N/kg OM; 40 vs 16 mg/L, Bi and Bt, respectively) and Spear grass (6.2 g N/kg OM; 29 vs 14 mg/L, Bi and Bt, respectively). In the same study, when forage was supplemented with N (30 g N/kg digestible OM), rumen ammonia concentrations were

similar between subspecies for cattle consuming Pangola grass (101 vs 112 mg/L, Bi and Bt, respectively) and Spear grass (78 vs 85 mg/L, Bi and Bt, respectively).

Thus, the subspecies difference in ruminal ammonia concentration is diet dependent, but becomes less substantial as N increases in the diet. Similarly, Hennessy et al. (1995) observed that the higher the Bi content of the genotype, the lower its N excretion on a low-N diet, and the higher its rumen ammonia concentration; thus, Bi cattle may have an increased ability to recycle ruminal N (Hunter and Siebert, 1985a; Hennessy et al., 2000).

Ruminal methane production

No differences in the proportion of CH₄ in fermentation gas between subspecies were reported by Hungate et al. (1960). *Bos indicus* cattle, however, frequently have greater numbers of rumen protozoa (Hennessy et al., 1995). O'Kelly and Spiers (1992b) observed greater ($P < 0.01$) protozoal population density in rumen fluid of Bi than Bt (44.5×10^6 vs 19.7×10^6 /L) at the same level of LQF intake. Because of the close association of protozoa with methanogens through interspecies H₂ transfer (Vogels et al., 1980; Finlay et al., 1994), it is reasonable to assume that Bi cattle would support greater CH₄ emission.

Summary

The abundance of LQF available for use in the beef cattle industry necessitates the development of strategies to improve its use and mitigate resulting CH₄ production. Protein supplementation strategies and ionophore inclusion have the potential to meet

these objectives. Characterization of the mechanism responsible for the differences observed in ability of Bi and Bt cattle to digest LQF may aid in the development of strategies effective in improving efficiency of forage nutrient utilization and mitigation of enteric CH₄ production. The research trials described in the following chapters were conducted in an effort to determine the effects of protein supplementation and ionophore inclusion on intake, digestion, ruminal fermentation, and microbial populations in cattle consuming LQF.

CHAPTER III

EFFECT OF LEVEL AND SOURCE OF SUPPLEMENTAL PROTEIN ON RUMINAL METHANE-PRODUCING ACTIVITY AND METHANOGEN AND PROTOZOA CONCENTRATION IN *BOS INDICUS* AND *BOS TAURUS* STEERS CONSUMING LOW-QUALITY FORAGE

Overview

Ten ruminally cannulated steers (5 *Bos indicus*, Bi, and *Bos taurus*, Bt) fed rice straw (4.4% CP) were used in concurrent 5 × 5 Latin squares to quantify effect of supplemental protein on CH₄-producing activity and methanogen and protozoa concentrations. Treatments were arranged as a 2 × 2 factorial plus control. Supplements (27% CP) were fed at 50 or 100 mg N/kg BW and low (L; 28%) or high (H; 72%) degradable intake protein (DIP). Rumen fluid was collected at feeding (0 h) and 4 h later. No subspecies differences were observed for any measurement ($P \geq 0.11$); however, to facilitate discussion, subspecies are discussed separately. Methane-producing activity in Bi steers declined after feeding ($P = 0.01$) from 2.37 to 1.65 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$. Methanogen concentration measured by most probable number (MPN) or PCR was not affected ($P \geq 0.36$) by protein supplementation or time after feeding in Bi steers. Protozoa concentration in Bi steers was greater with L-DIP ($P = 0.07$) than H-DIP at h 0 (4.34 vs 3.96 log₁₀ cells/mL). After feeding, protozoa concentration in Bi steers increased ($P = 0.05$) from 3.88 to 4.34 log₁₀ cells/mL. During h 4, lower ($P = 0.07$; 4.16 vs 4.53 log₁₀ cells/mL) protozoa concentrations were observed in Bi

supplemented 50 vs 100 mg N/kg BW. Similar to Bi, methane-producing activity in Bt steers tended to decline after feeding ($P = 0.09$) from 2.27 to 1.84 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$. Unlike Bi, however, MPN of methanogens in Bt tended to increase after feeding ($P = 0.08$) from 6.37 to 6.87 \log_{10} cells/mL. After feeding (h 4), supplementation increased PCR measured methanogen and protozoa concentrations (\log_{10} cells/mL) in Bt ($P \leq 0.03$) from 6.69 to 7.35 and 3.88 to 4.34, respectively. Results suggest that CH_4 -production may not be directly associated with the concentration of methanogens in the rumen.

Introduction

Low-quality forages (LQF; $\leq 7\%$ CP) are an important nutrient source used to maintain beef cattle throughout the world. Symbiosis between ruminants and their resident ruminal microbial population allows effective utilization of fibrous (e.g., cellulosic) materials present in LQF. Methane production is influenced by the nature of the carbohydrate digested; CH_4 production per gram of cellulose digested is five times that per gram of soluble residue digested (Moe and Tyrrell, 1979). Enteric CH_4 production is greater with consumption of LQF than high quality forage, because of its high fiber content. Development of strategies allowing more efficient use of LQF is crucial, not only for reducing economic costs, but also for alleviating the environmental burden associated with CH_4 emission. Supplemental protein is often provided to enhance intake and digestion of these forages to maintain animal performance and to optimize its use (Köster et al., 1996; Cochran et al., 1998; Wickersham et al., 2008a,b).

Beef cattle production systems in the southern United States often take advantage of *Bos indicus* (Bi) influenced cattle because of their tolerance for high ambient temperatures, humidity, and intense sunshine (Turner, 1980). *Bos taurus* (Bt) cattle, having evolved in cooler, more temperate climates (Howes et al., 1963), are not as well adapted to the climatic conditions prevalent in the tropics and subtropics. Comparisons of Bi and Bt cattle reveal that Bi are able to ferment N-deficient diets more rapidly than Bt (Hunter and Siebert, 1985a).

Although it is recognized that diet can affect diversity and concentration of a wide range of bacterial species in the rumen (Kocherginskaya et al., 2001; Popova et al., 2013; Ramsak et al., 2000; Tajima et al., 1999; Tajima et al., 2000; Whitford et al., 2001), limited information is available concerning concentrations of methanogenic populations or CH₄-producing activity with regard to supplemental protein level or source or the differences between the subspecies. Accordingly, the objectives of this study were to determine the effect of level and source of supplemental protein on ruminal CH₄-producing activity and methanogen and protozoa concentrations in Bi and Bt steers fed LQF.

Materials and methods

Animals and sampling

Experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University and included the use of anesthesia when surgical procedures were performed. The experiment was described in detail by Weldon (2013).

Briefly, ten steers (5 each: Bt, 303 ± 10 kg initial BW and Bi, 323 ± 28 kg initial BW) were fitted with ruminal and proximal duodenal (double- L shaped; Streeter et al., 1991) cannulas and assigned to separate 5×5 Latin squares by subspecies. Steers were housed in an enclosed, climate controlled barn with continuous lighting. Steers received a subcutaneous vitamin injection (3mL/animal; Vitamin AD Injection, Sparhawk Laboratories, Inc., Lenexa, KS) as a precaution against deficiency at onset of trial. Animals had continuous access to fresh water and commercial trace mineral-salt blocks ($\geq 96\%$ NaCl, 1.00% S, 0.15% Fe, 0.25% Zn, 0.30% Mn, 0.009% I, 0.015% Cu, 0.0025% Co, and 0.001% Se; United Salt Corporation, Houston, TX). The basal diet consisted of a low-quality rice straw (Table B1) coarsely chopped through a 76 mm \times 76 mm screen and offered daily at 130% of the previous 3 d average consumption.

Treatments were arranged as a 2×2 factorial plus an unsupplemented control (CON). The first factor was level of protein; 50 or 100 mg N/kg BW daily. The second factor was source; low DIP (L) supplement: 100% DDG (27% DIP) and high DIP supplement (H): a mixed protein supplement consisting of 69.5% wheat middlings, 30% SBM, and 0.5% urea (73% DIP).

The experiment consisted of five 15 d periods allowing 9 d for adaptation to treatment, 5 d for measurement of intake and digestion, and 1 d for determination of duodenal flow, ruminal fermentation, plasma urea N and rumen fluid sampling. Steers remained in individual pens (2.1 m \times 1.5 m) the first five days of each period and were moved to individual metabolism crates for the remainder of adaptation and throughout the collection period. Samples of rumen fluid were removed immediately prior to

feeding and 4 h later from four locations within the dorsal and ventral sac of the rumen. Collected rumen contents were squeezed through four layers of cheesecloth into insulated containers. Containers were filled, then capped to reduce oxygen exposure and immediately transported to the laboratory. In the laboratory, rumen fluid was blended using an IKA Lab Egg (Wilmington, NC) under a steady stream of oxygen-free CO₂ and aliquots were apportioned for measurement of ruminal CH₄-producing activity and concentrations of methanogens and protozoa. Additional parameters measured including forage intake and digestion, N retention, as well as ruminal NH₃, plasma urea N, pH, and volatile fatty acid concentrations, are described by Weldon (2013) while rate of ruminal NH₃ production and concentrations of amino acid-utilizing and trypticase-metabolizing bacteria are described in Chapter IV.

***In vitro* ruminal CH₄-producing activity**

In vitro ruminal CH₄-producing activity was determined by incubation of 5 mL freshly collected rumen fluid with 5 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 60mM sodium formate and 0.2g finely ground alfalfa as described by Anderson et al. (2006). Triplicate 18 × 150 mm crimp top culture tubes flushed with 50% H₂- 50% CO₂ were sealed using rubber stoppers and aluminum crimps and incubated 3h at 39°C. Upon conclusion of incubation period, a 1.0 mL gas sample was removed from the headspace of each tube and analyzed for CH₄ by gas chromatography on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a HaySep Q column (60°C, 25 mL/min of Argon carrier gas). Gas displacement was measured on all samples using a 20cc BD

Multifit glass syringe (Zone 3) with BD Luer-Lok tip (Becton, Dickinson and Company, Franklin Lakes, NJ). Methane-producing activity was calculated using the ideal gas law equation.

Determination of methanogens by Most Probable Number

Most probable number (MPN) of methanogens was determined using a three-tube Most Probable Number method (AOAC, 1980). Triplicate sets of tubes containing Balch 1 medium (Balch media as designated by Balch et al., 1979, Table 1) as modified by Saengkerdsut et al. (2007) were inoculated in series with 1 to 10^{-7} mL freshly collected and strained rumen fluid from each steer. Each tube was flushed with 80% H₂ - 20% CO₂ gas mixture at two atmospheres of pressure and incubated at 39°C for 20 days. On d 20 a 1.0 mL gas sample was removed from the headspace of each tube and analyzed for CH₄ on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a HaySep Q column (60°C, 25 mL/min of Argon carrier gas). Methanogen growth was assumed to have occurred in tubes having a positive CH₄ peak.

Determination of methanogen and protozoa concentration by PCR

Rumen fluid samples were mixed in a final concentration of 70% ethanol and frozen at -80°C. Upon completion of study, samples were shipped to the University of Vermont (Burlington, Vermont, USA) where they were stored at 4°C until commencement of DNA extraction.

DNA extraction

Samples were fully thawed for DNA extraction. DNA was extracted from a 0.25 g aliquot of each individual sample using the repeated bead-beating plus column (RBB + C) method (Yu and Morrison, 2004) and QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, Maryland). Samples were homogenized using zirconia beads for 3 min, then incubated with lysis buffer (Yu and Morrison, 2004) at 70°C for 15 min, followed by centrifugation at 4°C for 5 min at 16,000 × *g*. After repeating this process a second time, supernatant was treated with an InhibiTEX tablet from the QIAGEN kit. Remainder of extraction process followed manufacturer's instructions. Final elutions were made to 200 µL of TE (Tris-EDTA, pH 8.0) buffer, and DNA was quantified using a NanoDrop 2000C Spectrophotometer (ThermoScientific, California). Purity of DNA extract was verified using gel electrophoresis to molecular weight. DNA extract was also PCR amplified on a CFX96 ThermoCycler (Bio-Rad, CA) using the iTaq kit (Bio-Rad, CA) to test DNA quality. All PCR results were run on a 1% agarose gel at 100 volts for 60 min and imaged on a ChemiDoc XRS+gel imager (Bio-Rad, CA) to determine correct PCR amplicon length prior to quantitative real-time PCR.

Quantitative real-time PCR analysis

For each microbial group (methanogenic archaea and ciliate protozoa), real-time PCR was performed on a CFX96 ThermoCycler (Bio-Rad, CA) with a C1000 real-time hood (Bio-Rad, CA) to calculate concentrations in each sample. Samples were analyzed using *mcrA-F* and *mcrA-R* primers targeting the methyl-coenzyme M reductase subunit for methanogenic archaea (Denman et al., 2007) and *P-SSSU-316F* and *P-SSSU-539R*

targeting the 18srRNA gene of ciliate protozoa (Sylvester et al., 2004). Each reaction contained 12.5 μ L of iQ SYBR Green Supermix kit (Bio-Rad, CA): 2.5 μ L of each primer (40mM; Table B2), 6.5 μ L ddH₂O, and 1 μ L of initial DNA extract diluted to approximately 10 ng/ μ L. External standards have been previously validated for methanogenic archaea (Denman et al., 2007) and ciliate protozoa (Sylvester et al., 2004). Methanogen protocol consisted of initial denaturing at 95°C for 15 min, then 40 cycles of: 95°C for 30s, 60°C for 30s, 72°C for 30s. Protozoa protocol consisted of initial denaturing at 94°C for 4 min, then 45 cycles of: 94°C for 30 s, 54°C for 30 s, 72°C for 1min. Both methanogen and protozoa protocol were followed by a melt curve with 0.5°C temperature increase every 10s from 60°C up to 95°C to check for contamination. Data were analyzed using the CFX Manager Software v1.6 (Bio-Rad, CA).

Statistical analysis

Determination of methanogens by MPN and determination of methanogens and protozoa by PCR were log transformed to normally distribute residuals. Methane producing activity and transformed MPN and PCR data were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included treatment, hour, treatment \times hour and period with animal \times treatment \times period and animal included as random effects. The repeated term was hour with animal \times treatment as the subject. Treatments were calculated using the LSMEANS option. A contrast was used for separation of means between Bi and Bt at h 0 and 4.

Subspecies were then analyzed separately. Methane producing activity, MPN of methanogens, and protozoa and methanogen concentrations measured by PCR were

analyzed using the MIXED procedure of SAS 9.3. Terms in the model included treatment, hour, treatment \times hour, and period. Treatment means were calculated using the LSMEANS option. Contrasts were used for separation of means at h 0 and 4. Contrasts included source \times level interaction and direct comparisons of levels of supplement (50 versus 100), source of DIP (H versus L), and CON versus supplemented treatments.

Results

No subspecies differences were observed for CH₄-producing activity, MPN of methanogens, or concentrations of methanogens or protozoa measured by PCR (Table B3); however to facilitate discussion of the data, subspecies are treated separately.

A treatment \times time after feeding interaction was not observed ($P \geq 0.70$) in Bi steers for any of the measured variables. *In vitro* ruminal CH₄-producing activity decreased ($P = 0.01$) from h 0 to 4 (from 2.37 to 1.65 $\mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$; Fig. B1). Methane-producing activity of Bi steers was not affected ($P \geq 0.22$) by treatment. Effects of treatment or time after feeding were not observed ($P \geq 0.36$) for concentration of methanogens measured by PCR. An effect of time after feeding was not observed ($P = 0.31$) for concentration of protozoa measured by PCR. Before feeding (h 0), L-DIP supplementation resulted in greater ($P = 0.07$; 4.34 vs 3.96 \log_{10} cells/mL) protozoa concentration than H-DIP supplementation (Table B4). After feeding (h 4), greater ($P = 0.05$) protozoa concentration (4.34 vs 3.88 \log_{10} cells/mL) was observed in protein supplemented than CON Bi steers. Furthermore, at h 4, there was a tendency ($P = 0.07$)

for lower protozoa concentrations (4.16 vs 4.53 log₁₀ cells/mL) for 50 vs 100 mg N/kg BW.

A treatment × time after feeding interaction was not observed ($P \geq 0.27$) in Bt steers for any of the measured variables. *In vitro* ruminal CH₄-producing activity tended to decrease ($P = 0.09$) from h 0 to 4 (from 2.27 to 1.84 μmol·mL⁻¹·h⁻¹). Methane-producing activity was unaffected ($P \geq 0.18$) by treatment. An effect of time after feeding was observed ($P = 0.08$) for MPN of methanogens with an increase (from 6.37 to 6.87 log₁₀ cells/mL) occurring from h 0 to 4 after feeding (Fig. B2). Most probable number of methanogens was not affected ($P \geq 0.15$) by treatment. An effect of time after feeding was not observed ($P = 0.86$) for methanogen concentration measured by PCR. After feeding (h 4), a greater ($P = 0.02$) methanogen concentration (7.35 vs 6.69 log₁₀ cells/mL) was observed in protein supplemented versus CON Bt steers when measured by PCR. An effect of time after feeding was not observed ($P = 0.92$) for concentration of protozoa measured by PCR. After feeding (h 4), greater ($P = 0.03$) protozoa concentration (4.08 vs 3.55 log₁₀ cells/mL) was observed in protein supplemented versus CON Bt steers.

Discussion

Solutions to alleviate economic and environmental costs associated with enteric CH₄ emissions from ruminants are actively sought. Protein supplementation provides an opportunity to stimulate intake and digestion of LQF, optimizing its utilization and potentially mitigating enteric CH₄-production. Brahman cattle were used to represent the

Bi subspecies, while Angus cattle were used to represent the Bt subspecies to evaluate the effect of different sources and levels of protein supplementation on CH₄-producing activity and methanogen concentrations in the rumen.

Methane-producing activity

Protein supplementation did not significantly affect CH₄-producing activity, increasing it 11% in supplemented Bi (from 1.85 to 2.05 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ for CON and supplemented Bi) and 7% in supplemented Bt (from 1.94 to 2.08 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ for CON and supplemented Bt). Furthermore, CH₄-producing activity was not different between the two levels or sources of supplemental protein. Although the direct effect of protein supplementation on methanogenesis is variable and unclear (Moss, 2000), previous work indicates that protein supplementation decreases CH₄ production as a % of DE in the rumen (Mehra et al., 2006) and per unit product (meat or milk; Moss, 1994; Moss et al., 2000). Mehra et al. (2006) observed a 31.6% decrease in CH₄ energy as a % of DE in buffalo consuming *ad libitum* wheat straw (3.62% CP) supplemented with 1.8 kg soybean meal (52.44% CP) versus control animals consuming only wheat straw. Similarly, Huque and Chowdhury (1997) observed a 32.3% reduction in *in vitro* CH₄ production (mL/g OM apparently fermented in the rumen) in growing bulls fed a urea and molasses mixture (10% CP) with rice straw (5.37% CP) versus the control diet of rice straw alone.

Time after feeding had a direct impact on CH₄-producing activity, however, the direction of impact is in contrast to previous work (Clapperton and Czerkawski, 1969; Johnson et al., 1994). Peaks in CH₄ production generally correspond with peaks in eating

activity (Lockyer and Champion, 2001). Methane production is generally observed to be greater soon after feeding (Clapperton and Czerkawski, 1969; Johnson et al., 1994) when the partial pressure of hydrogen is highest and when substrate availability is greatest. However, a decrease was observed from h 0 to 4 in both Bi and Bt steers (30.4% and 18.9%, respectively). A reduction of methanogenesis in the rumen is often accompanied by an increase in propionate production (DeMeyer et al., 1969; Prins, 1970; Van Nevel et al., 1971). It is suggested by Van Nevel et al. (1974) that when methane formation is inhibited, the excess of hydrogen gas is shunted away to other reductive processes such as propionate production. The inverse relationship between the two is therefore based on a competition of both systems for metabolic hydrogen (Van Nevel et al. 1974). Molar percentage of propionate increased in the present study from h 0 to 4 in both Bi ($P < 0.01$; from 17.04 to 17.60%) and Bt ($P = 0.02$; from 17.35 to 17.70%) steers (Weldon, 2013). This observation supports the concept of an inverse relationship between propionate and methane production.

Methanogens

A 7.9% increase in MPN of methanogens was observed in Bt steers from h 0 to 4. Although unexpected, because CH₄-producing activity decreased from h 0 to 4, this observation is in agreement with others (Firkins and Yu, 2006; Nhan et al. 2012), suggesting that a reduction of methane production is not necessarily associated with a reduced number of methanogens in the rumen. Further work is necessary to determine the relationship between number of methanogens present in the rumen and CH₄-producing activity.

As expected, methanogen concentration when measured by PCR, yielded different results than when measured by MPN; MPN measures viable cells, while PCR can measure both live and dead cells. When measured by PCR, protein supplementation increased methanogen concentration during h 4 by 9.9 % in Bt steers, likely due to greater N-availability.

Ciliate protozoa

Association of methanogenic archaea with rumen ciliates is a common feature of anaerobic systems (Vogels et al., 1980) and is of value to both organisms, enabling interspecies hydrogen transfer (Wolin, 1974). Ciliate protozoa depend on a hydrogen evolving fermentation, but benefit from hydrogen removal because of its inhibitory effect on their metabolism (Sharp, et al., 1998). Methanogens attached to protozoa derive H₂ directly from specialized organs called hydrogenosomes (Hungate, 1966). The H₂ is used by methanogens for the energy-yielding reduction of CO₂ to CH₄.

Although the association of methanogens with the outer surface of ciliates was discovered long ago (Wolin, 1974; Vogels et al., 1980), more recent research (Finlay et al., 1994) has revealed methanogens living inside rumen ciliates. The first documented case of methanogens living inside rumen ciliates was provided by Finlay et al. (1994) when intracellular bacteria free in the cytoplasm of the most abundant ciliate species, *Dasytricha ruminantium* and *Entodinium* spp. were observed. It was suggested that all of the intracellular bacteria were likely methanogens, accounting for approximately 1-2 % of host ciliate volume. Additionally, defaunation of protozoa from the rumen has been associated with a 30-45 % reduction in CH₄ (Jouany, 1991; Ushida et al., 1997),

confirming the close relationship between ruminal methanogens and ciliate protozoa. Protozoa concentration in the present study was measured by PCR and evaluated in an effort to explain the increase in MPN of methanogens from h 0 to 4. Protozoa concentration was unaffected by time after feeding, and did not explain this occurrence.

Prior to feeding, h 0, protozoa concentration in Bi steers differed in response to source of supplemental protein. *Bos indicus* steers consuming H-DIP had 8.8 % lower protozoal concentration than those consuming L-DIP. This observation is in contrast to the original hypothesis that greater DIP would support greater protozoal concentration because of the involvement of protozoa in degradation of dietary proteins (Hino and Russell, 1987; Hsu et al., 1991; Jouany, 1996; Han et al. 1999).

Protozoa, having high deaminase activities (Hino and Russell, 1987), are actively involved in the degradation of dietary proteins in the rumen (Jouany, 1996). After feeding, greater substrate availability from protein supplementation would be expected to support a larger population of protozoa than the control diet. Accordingly, protein supplementation resulted in greater protozoa concentration at h 4 by 11.8% in Bi and 15.0 % in Bt compared with control steers. Additionally, 4 h after feeding, Bi steers consuming 100 mg N/kg BW had 8.9% greater protozoa concentration than those consuming 50 mg N/kg BW. A negative consequence of increased protozoa concentration, however, is the reduced flow of protein to the small intestine. Hsu et al. (1991) reported defaunation increased crude protein flow to the duodenum and reduced ruminal NH₃-N concentration. Similarly, Ankrah et al. (1990) and Damry (2009) observed greater rumen NH₃-N concentrations in faunated than defaunated

lambs. Greater rumen $\text{NH}_3\text{-N}$ is due to increased protein degradation (Veira, 1986). Accordingly, Weldon (2013) evaluated ruminal $\text{NH}_3\text{-N}$ in the present study and observed a 44.5% increase (from 1.46 to 2.11 mM; $P < 0.01$) from h 0 to h 4 suggesting reduced N-flow to the duodenum at h 4, when protozoa concentration is greater, than h 0.

CHAPTER IV

**EFFECT OF LEVEL AND SOURCE OF SUPPLEMENTAL PROTEIN ON
RATE OF RUMINAL AMMONIA PRODUCTION AND CONCENTRATIONS
OF AMINO ACID-UTILIZING AND TRYPTICASE-METABOLIZING
BACTERIA IN *BOS INDICUS* AND *BOS TAURUS* STEERS CONSUMING
LOW-QUALITY FORAGE**

Overview

Ten ruminally cannulated steers (5 *Bos indicus*, Bi, and *Bos taurus*, Bt) fed rice straw (4.4% CP) were used in concurrent 5 × 5 Latin squares to quantify effect of supplemental protein on *in vitro* rate of ruminal NH₃ production and concentrations of amino acid-utilizing and trypticase-metabolizing bacteria. Treatments were arranged as a 2 × 2 factorial plus control. Supplements (27% CP) were fed at 50 or 100 mg N/kg BW and low (L; 28%) or high (H; 72%) degradable intake protein (DIP). Rumen fluid was collected at feeding and 4 h later. No subspecies differences were observed for any measurement ($P \geq 0.28$). A slower ($P = 0.04$) rate of ruminal NH₃ production was observed at h 4 in Bt steers fed L-DIP (33.59) than H-DIP (33.6 and 50.6 $\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, respectively) Bt steers. At h 0, most probable number of amino acid-utilizing bacteria (\log_{10} cells/mL) in protein supplemented Bt steers was greater ($P < 0.01$) than control steers (6.03 vs 5.25). Most probable number was greater ($P = 0.09$) at h 0 for Bt supplemented 100 vs 50 mg N/kg BW (6.21 vs 5.85). A time effect ($P \leq 0.01$) on trypticase-metabolizing bacteria occurred in Bi and Bt steers with fewer \log_{10} cfu/mL

present 4 h after feeding versus 0 h (7.06 vs 7.52). Fewer ($P = 0.04$) trypticase-metabolizing bacteria were present at h 0 in H-DIP than L-DIP Bi steers (7.27 vs 7.76 \log_{10} cfu/mL). Protein supplemented Bt steers had fewer ($P = 0.02$) trypticase-metabolizing bacteria present at h 0 than control steers (7.38 vs 8.02 \log_{10} cfu/mL). Irrespective of subspecies, microbial N-metabolizing populations responded more to H-DIP than L-DIP supplementation.

Introduction

Low-quality forages (LQF; $\leq 7\%$ CP) provide a valuable source of nutrients to ruminants worldwide. Considerable research has been directed toward the development of strategies to overcome the inadequate supply of N provided to the rumen by LQF. Protein supplementation provides an opportunity to stimulate microbial growth, ultimately improving forage intake, digestion, and animal performance (Del Curto et al., 1990; Köster et al., 1996). Supplements high in degradable intake protein (DIP) are typically recommended because they directly address the ruminal N deficiency that exists when LQF are consumed. Ruminal microorganisms, such as hyperammonia-producing bacteria (HAP; *Peptostreptococcus anaerobius*, *Clostridium stricklandii*, and *Clostridium aminophilum*), degrade DIP into NH_3 . Hyperammonia-producing bacteria have high specific activities of deamination, with an average 20-fold higher specific activity for NH_3 production compared with other ruminal NH_3 producers (Chen and Russell, 1989). Excess NH_3 is absorbed across the rumen wall, transported to the liver and detoxified to urea, which may be excreted in urine or recycled back into the rumen

and utilized as a source of N for microbial growth (Wickersham et al., 2009). Because undegradable intake protein (UIP) is not ruminally degraded, the use of these supplements to meet ruminal N requirements and improve utilization of LQF is dependent exclusively on an indirect supply of N to the rumen through urea recycling (Bandyk et al., 2001; Wickersham et al., 2009).

The effect of protein supplementation on intake, digestion, and ruminal fermentation parameters of Bi and Bt cattle consuming LQF is well documented (Hunter and Siebert, 1987; Hennessy et al., 1995, 2000; Hegarty, 2004) and reveals that Bi cattle are able to ferment nitrogen-deficient diets more rapidly than Bt cattle. In these studies, higher ruminal NH₃ concentrations are observed in Bi than Bt cattle (Hunter and Siebert, 1985a, 1987; Hennessy et al., 1995, 2000) and may be associated with differences between subspecies in ruminal concentration of HAP bacteria. This study was designed to compare the effects of source and level of protein supplement on rate of ruminal NH₃ production and to isolate and enumerate concentrations of amino acid-utilizing and trypticase-metabolizing bacteria in Bi and Bt steers fed LQF in an effort to explain the difference in ruminal NH₃ concentration that occurs across subspecies.

Materials and methods

Animals and sampling

Experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University, and included the use of anesthesia when surgical procedures were performed. The experiment was described in detail by Weldon (2013).

Briefly, ten steers (5 each: *Bos taurus*, Bt, 303 ± 10 kg initial BW and *Bos indicus*, Bi, 323 ± 28 kg initial BW) were fitted with ruminal and proximal duodenal (double-L shaped; Streeter et al., 1991) cannulas and assigned to separate 5×5 Latin squares by subspecies. Steers were housed in an enclosed, climate controlled barn with continuous lighting. Steers received a subcutaneous vitamin injection (3mL/animal; Vitamin AD Injection, Sparhawk Laboratories, Inc., Lenexa, KS) as a precaution against deficiency at onset of trial. Animals had continuous access to fresh water and commercial trace mineral-salt blocks ($\geq 96\%$ NaCl, 1.00% S, 0.15% Fe, 0.25% Zn, 0.30% Mn, 0.009% I, 0.015% Cu, 0.0025% Co, and 0.001% Se; United Salt Corporation, Houston, TX). The basal diet consisted of a low-quality rice straw (Table C1) coarsely chopped through a 76 mm \times 76 mm screen and offered daily at 130% of the previous 3 d average consumption.

Treatments were arranged as a 2×2 factorial plus an unsupplemented control (CON). The first factor was level of protein; 50 or 100 mg N/kg BW daily. The second factor was source; low DIP (L) supplement: 100% DDG (27% DIP) and high DIP supplement (H): a mixed protein supplement consisting of 69.5% wheat middlings, 30% SBM, and 0.5% urea (73% DIP).

The experiment consisted of five 15 d periods allowing 9 d for adaptation to treatment, 5 d for measurement of intake and digestion, and 1 d for determination of duodenal flow, ruminal fermentation, plasma urea N and rumen fluid sampling. Steers remained in individual pens (2.1 m \times 1.5 m) the first five days of each period and were moved to individual metabolism crates for the remainder of adaptation and throughout

the collection period. Samples of rumen fluid were removed immediately prior to feeding and 4 h later from four locations within the dorsal and ventral sac of the rumen. Collected rumen contents were squeezed through four layers of cheesecloth into insulated containers. Containers were filled, then capped to reduce oxygen exposure and immediately transported to the laboratory. In the laboratory, rumen fluid was blended using an IKA Lab Egg (Wilmington, NC) and aliquots were apportioned for measurement of ruminal rate of ruminal NH₃-producing activity and concentrations of amino acid-utilizing and trypticase-metabolizing bacteria. Additional parameters measured including forage intake and digestion, N retention, as well as ruminal NH₃, plasma urea N, pH, and volatile fatty acid concentrations, are described by Weldon (2013) while CH₄-producing activity and concentrations of methanogens and protozoa are described by Bell et al. (2014).

Rate of *in vitro* ruminal ammonia production

Ammonia-producing activity was determined by incubation of 2 mL of freshly collected rumen fluid with 6 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 2% (wt/vol) Bacto trypticase (Becton, Dickinson and Company, Sparks, MD). Triplicate 18 × 150 mm crimp top culture tubes flushed with 100% CO₂ were sealed using rubber stoppers with aluminum crimps and incubated at 39°C. Immediately prior to (h 0) and after 6 h incubation a 0.5 mL sample was removed and frozen for determination of ruminal NH₃ production.

For analysis, frozen samples were thawed and centrifuged at 18,000 × *g* for 10 min at 22°C. Ammonia was assayed colorimetrically using Berthelot's reaction according to

Chaney and Marbach (1962). Rates of NH_3 production were calculated by dividing the difference between 6 and 0 h concentrations by 6 h incubation time; concentrations were obtained using a Tecan Infinite M200 Pro Micro Plate Reader and Tecan Megellan Software (Tecan, Salzburg, Austria).

Most Probable Number determination of amino acid-utilizing bacteria

Most probable number of amino acid-utilizing bacteria was determined by inoculating serial 10-fold dilutions of strained freshly collected rumen fluid in a basal medium based on Eschenlauer et al. (2002) except containing slightly lower salt concentrations as indicated, per L: 225 mg K_2HPO_4 , 225 mg KH_2PO_4 (monobasic), 450 mg $(\text{NH}_4)_2\text{SO}_4$, 450 mg NaCl, 45 mg $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 22 mg $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$. Resazurin, cysteine-HCl, Na_2CO_3 , microminerals and vitamins were included as described by Eschenlauer et al. (2002) except lipoic acid and Vitamin B_{12} were included at half concentration. Bacto Casamino Acids (15 g/liter; Becton, Dickinson and Company, Sparks, MD) were added as the energy and carbon source for bacterial growth. Medium was kept under a continuous flow of 100% CO_2 , adjusted to pH 7.0, boiled, then 4.5 mL was added to triplicate 18×150 mm crimp top culture tubes before autoclaving. Tubes were incubated at 39°C for 7 d. Growth was determined by measuring optical density (OD) at 600 nm on a Spectronic 20D+ spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Growth was assumed to have occurred in inoculated tubes when OD increased by at least 0.1 absorbance units from that of uninoculated controls. This value is the lower cut off value used by Eschenlauer et al. (2002).

Enumeration of trypticase-metabolizing bacteria

Trypticase-metabolizing bacteria were enumerated using an agar medium based on Eschenlauer et al. (2002) with casamino acids in the basal medium described above being replaced with Bacto Trypticase Peptone (15 g/Liter) and the addition of 2% (wt/vol) Bacto Agar (Becton, Dickinson and Company). Moreover, Na₂CO₃ was omitted from the agar medium to accommodate its use in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing CO₂:N₂:H₂ (5:90:5) rather than 100% CO₂. Plates were solidified and dried overnight at room temperature and equilibrated to the anaerobic gas phase within the chamber before use.

Strained freshly collected rumen fluid diluted in 9 mL of anaerobic dilution solution (Bryant and Burkey, 1953), modified by omitting Na₂CO₃, was used to inoculate anaerobic plates by spreading 0.1 mL of 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions. Plates were incubated at 39°C for 7 days at which time all discernible colonies were counted.

Statistical analysis

Most probable numbers of amino acid-utilizing bacteria and total CFUs of trypticase-metabolizing bacteria were log transformed to normally distribute residuals. Rate of ruminal NH₃ production and transformed MPN and CFU data were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included treatment, hour, treatment × hour and period with animal × treatment × period and animal included as random effects. The repeated term was hour with animal × treatment as the subject. Treatments were calculated using the LSMEANS option. A contrast was used for separation of means between Bi and Bt at h 0 and 4.

Subspecies were then analyzed separately. Rates of ruminal NH₃ production were analyzed using the MIXED procedure of SAS 9.3. Terms in the model included treatment, hour, treatment × hour, and period. Treatment means were calculated using the LSMEANS option. Contrasts were used for separation of means at h 0 and 4. Contrasts included source × level interaction and direct comparisons of levels of supplement (50 versus 100), source of DIP (H versus L), and CON versus supplemented treatments.

Results

No subspecies differences were observed for rate of ruminal NH₃ production or concentration of amino acid-utilizing or trypticase metabolizing bacteria (Table C2); however to facilitate discussion of the data, subspecies are treated separately.

A treatment × time after feeding interaction was not observed ($P \geq 0.61$) in Bi steers for any of the measured variables. Effects of treatment or time after feeding were not observed ($P \geq 0.29$) for rate of ruminal NH₃ production or MPN of amino acid-utilizing bacteria. An effect of time after feeding was observed ($P < 0.01$) for trypticase-metabolizing bacteria with fewer log₁₀ cfu/mL present 4 h after feeding versus 0 h (7.1 vs 7.5, respectively). At h 0, fewer ($P = 0.04$) trypticase-metabolizing bacteria were present in Bi steers supplemented with H-DIP than L-DIP (7.27 vs 7.75 log₁₀ cfu/mL).

Main effects of time after feeding or a treatment × time after feeding interaction were not observed ($P \geq 0.41$) for rate of ruminal NH₃ production in Bt steers. A source × level interaction was observed (Fig. C1) on rate of ruminal NH₃ production in Bt steers during

h 4 with reduced rates observed with increasing level of L-DIP and increased rates observed with increasing level of H-DIP. Also during h 4, ammonia production was faster in Bt steers consuming H-DIP versus L-DIP. A treatment \times time interaction ($P = 0.04$) occurred in Bt steers for MPN of amino acid-utilizing bacteria with CON and 50 L-DIP increasing over time while both levels of H-DIP and 100 L-DIP decreased over time (Fig. C2). At h 0, MPN in protein supplemented Bt steers was greater ($P < 0.01$) than control steers (6.03 vs 5.25 \log_{10} cells/mL, respectively). Also in Bt at h 0, steers supplemented with 50 vs 100 mg N/kg BW were observed to have fewer ($P = 0.09$) amino acid-utilizing bacteria (5.85 vs 6.21 \log_{10} cells/mL). A treatment \times time after feeding interaction was not observed ($P \geq 0.46$) for trypticase-metabolizing bacteria. An effect of time after feeding was observed ($P < 0.01$) for trypticase-metabolizing bacteria with fewer \log_{10} cfu/mL present 4 h after feeding versus 0 h (7.0 vs 7.5, respectively; Fig. C3). Protein supplemented Bt steers were observed to have fewer ($P = 0.02$) trypticase-metabolizing bacteria present at h 0 than control steers.

Discussion

Brahman cattle were used to represent the Bi subspecies, while Angus cattle were used to represent the Bt subspecies to evaluate the effect of different sources and levels of protein supplementation on rate of NH_3 production, MPN of amino acid-utilizing bacteria, and trypticase-metabolizing bacteria. No subspecies differences were observed for any of the measured parameters; however, to facilitate discussion of the data, subspecies are treated separately.

Protein supplementation is a widespread practice used to overcome the limitations associated with forage quality. Dietary protein is degraded in the rumen by proteases, peptidases, and deaminases to produce peptides, amino acids, and ammonia, respectively (Bach et al., 2005). Hyperammonia-producing bacteria metabolize amino acids to produce energy, an inefficient use of dietary protein that results in increased ammonia production and decreased dietary protein flow to the small intestine. In ruminants, as much as 50% of the dietary protein is converted to ammonia by microorganisms (Nolan, 1975). Often, this process results in excessive ruminal ammonia production representing an inefficiency in ruminant metabolism and the excretion of excess N in the urine.

Bos taurus

After feeding (h 4), increasing protein supplementation from 50 to 100 mg N/kg BW increased rate of NH₃ production by 37.9% in steers consuming H-DIP, but had almost no effect (0.8% decrease) on NH₃ production in steers consuming L-DIP. At h 4, H-DIP supplementation resulted in 49.9% faster rate of NH₃ production than L-DIP. Because DIP is converted to ammonia in the rumen, directly addressing the nutrient requirements of ruminal bacteria (Allison, 1969), increases in ruminal ammonia-N (RAN) concentration are apparent with its supplementation (Bandyk et al. 2001; Klevesahl et al., 2003; Wickersham et al., 2004, 2008b). Wickersham et al. (2008b) observed a linear increase ($P < 0.01$; from 0.27 to 3.66 mM) in RAN as level of DIP (casein) supplementation increased from 0 to 177 mg N/kg BW. With UIP supplementation, however, only small increases are observed (Wickersham et al., 2009), as it is undegradable and does not directly address nutrient requirements. When cattle were

supplemented with increasing levels of UIP (Wickersham et al., 2008a) the increase was not as substantial (0.08 to 0.55 mM when UIP increased from 0 to 186 mg N/kg BW).

Although much of the protein that enters the rumen is fermented, Bladen et al. (1961) noted that many pure cultures of ruminal bacteria were unable to produce NH₃. Strains of *Bacteroides* (now *Prevotella*) *ruminicola*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Eubacterium ruminantium*, and *Butyrivibrio fibrisolvens* were found to produce some NH₃, but even with a 96 hr incubation period, less than 25% of the casein hydrolysate was converted to NH₃ (Bladen et al., 1961). In the 1980s, enrichment cultures including high concentrations of protein hydrolysate yielded three ruminal bacterial species with specific activities of NH₃ production 18 to 39 times higher than previously identified species (Russell et al., 1988). These bacteria, did not utilize carbohydrates, rather, they utilized amino acids as their sole carbon and energy source, and are known as “obligate amino acid-fermenting” or “hyperammonia-producing bacteria” (Russell et al., 1988). Characterized by high specific activities of NH₃ production, these bacteria were later determined, by 16S rRNA sequence analyses, to be *Peptostreptococcus anaerobius*, *Clostridium stricklandii*, and a new species designated *Clostridium aminophilum* (Paster et al., 1993). Yang and Russell (1993) used most-probable number estimates to determine that these obligate amino acid-utilizing bacteria accounted for less than 10% of the total ruminal bacteria count; but calculated that these species are capable of deaminating over 25% of protein in feeds (Krause and Russell, 1996).

Properties of bacteria isolated on the basis of ability to grow on trypticase or Casamino Acids have important implications for understanding the biochemistry and microbial ecology of NH₃ production in the rumen of cattle. Casamino Acids is an acid hydrolysate of casein which contains mostly free amino acids while trypticase is an enzymatic digest of casein containing peptides and relatively few free amino acids.

Plate counts on medium with trypticase as the sole energy source resulted in greater colony numbers than MPN counts using Casamino Acids (7.25 vs 5.85 log₁₀ cells·mL⁻¹) in Bt steers.

Effects of protein supplementation were observed for MPN of amino acid-utilizing bacteria and trypticase-metabolizing bacteria. A decrease in MPN of amino-acid utilizing bacteria from h 0 to 4 was observed in steers consuming both levels of H-DIP and 100 L-DIP, while an increase was observed in CON and 50 L-DIP. Prior to feeding (h 0), a 14.8% greater amino-acid utilizing bacteria concentration was observed in protein supplemented Bt steers. Also at h 0, Bt steers receiving 100 mg N/kg BW were observed to have 6.1% greater concentration of amino-acid utilizing bacteria than those receiving 50 mg N/kg BW.

Colonies of trypticase-metabolizing bacteria in Bt steers declined 6.8% from 0 to 4 h after feeding. Prior to feeding (h 0), colonies of trypticase-metabolizing bacteria in protein supplemented Bt steers were 7.9% lower than for CON steers.

Bos indicus

Ruminal NH₃-N concentration was increased ($P < 0.01$) by protein supplementation versus the control in the present study (1.08 vs 2.35 mM; Weldon, 2013). However,

protein supplementation source or level did not affect rate of NH₃ production.

Additionally, time after feeding did not affect rate of NH₃ production. Ruminal NH₃-N concentration peaked 2 h after feeding (Weldon, 2013) for all treatments. It is reasonable to assume that most of the available free amino acids were depleted before 4 h after feeding and that the 0 and 4 hour sampling times used to measure rate of NH₃ production were simply not adequate time points to capture the increased rate of NH₃ production that likely occurred to produce the increased RAN concentration.

Similar to Bt, plate counts on medium with trypticase as the sole energy source resulted in greater colony numbers than MPN counts using Casamino Acids in Bi (7.32 vs 6.03 log₁₀ cells·mL⁻¹).

Unlike the Bt steers, effects of protein supplementation at any source or level were not observed in Bi for MPN of amino acid-utilizing bacteria or trypticase-metabolizing bacteria. Time after feeding did not affect MPN of amino acid-utilizing bacteria. When the 2 h peak in RAN is considered, it is clear that protein degradation is occurring quickly. It is possible that most of the free amino acids were depleted before the 4 h sampling time, and this prevented detection of an effect of protein supplementation on MPN of amino acid-utilizing bacteria when samples are collected at h 4. Observations by Farmer et al. (2004) support this hypothesis. Most probable number of peptide and amino acid-fermenting bacteria peaked 2 h after supplementation and returned to nadir by 12 h after supplementation in steers consuming tallgrass prairie hay and supplemented with a 42% CP (0.36% BW/d; DM basis) supplement (Farmer et al.,

2004). Prior to feeding (h 0), however, 6.3% fewer trypticase-metabolizing bacterial colonies were observed in H-DIP versus L-DIP Bi steers.

CHAPTER V

EFFECT OF MONENSIN INCLUSION ON INTAKE, DIGESTION, AND RUMINAL FERMENTATION PARAMETERS IN *BOS INDICUS* AND *BOS TAURUS* STEERS CONSUMING BERMUDAGRASS HAY

Overview

Effects of monensin inclusion and cattle subspecies on the utilization of bermudagrass hay (13.7% CP) were evaluated using ruminally cannulated steers (5 *Bos indicus*, Bi and 5 *Bos taurus*, Bt; 398 kg BW). Subspecies were concurrently subjected to a two period, two treatment crossover design. Treatments consisted of 0 (CON) or 200 (MON) mg·hd⁻¹ monensin fed daily in 0.91 kg DDGS; monensin provided by Rumensin® 90 (Elanco Animal Health, Greenfield, IN). Steers were group housed during adaptation periods and moved to individual covered pens for sampling (d 17-25 and 41-42). Periods were 70 d in length allowing 20 d adaptation, 22 d sample collection, and 28 d withdrawal between periods. Hay, ort, and fecal grab samples were collected d 21-25 for determination of intake and digestion. Ruminal fluid was collected with a suction-strainer 0, 2, 4, 8, and 12 h after feeding on d 42 for analysis of pH, VFA, and ruminal NH₃-N (RAN). Additionally at h 2, ruminal contents were squeezed through four layers of cheesecloth into insulated containers for determination of rate of NH₃ production and CH₄-producing activity. No subspecies × monensin interactions were observed ($P \geq 0.14$). Monensin tended to increase ($P = 0.08$) forage OM intake from 19.5 to 20.3 g/kg BW. *Bos taurus* steers had greater ($P = 0.04$) total digestible OM

intake than Bi steers (14.3 vs 12.2 g/kg BW). There was an effect of time after feeding ($P \leq 0.01$) on pH, total VFA, acetate:propionate, and molar percentages of acetate and propionate. Total VFA concentration was greater ($P = 0.01$) in CON vs MON steers (66.5 vs 62.0 mM). Monensin decreased the molar percentage of acetate ($P = 0.02$) from 72.5% to 71.2% and increased the molar percentage of propionate ($P < 0.01$) from 16.9% to 18.7%, resulting in a reduced ($P < 0.01$; from 4.34 to 3.85) acetate:propionate ratio. Although CH₄-producing activity was not significantly different ($P = 0.19$) between CON and MON, monensin feeding resulted in a 15.8% reduction in CH₄-producing activity. *Bos indicus* steers tended to have greater ($P = 0.07$) CH₄-producing activity than Bt steers (21.4 vs 16.6 $\mu\text{mol CH}_4 \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$). Monensin had no effect ($P \geq 0.19$) on pH, RAN, or rate of NH₃ production. A subspecies \times hour after feeding interaction was observed for RAN with Bt having greater RAN at h 0 and 4, while Bi had greater RAN at h 2, 8 and 12. Overall, monensin decreased the acetate:propionate ratio and increased forage intake. *Bos indicus* consumed less digestible OM and had greater CH₄-producing activity compared to Bt steers.

Introduction

Although it is well established that monensin improves feed efficiency of feedlot and stocker cattle (Horn et al., 1981; Goodrich et al., 1984; Lana et al., 1997; Beauchemin and McGinn, 2005; Guan et al., 2006), limited data is available concerning the effect of monensin on feed efficiency of grazing cattle (Oliver, 1975; Lemenegre et al., 1978a; Crosthwait et al., 1979; Rouquette et al., 1980; Ellis et al., 1983; Ward et al., 1990a;

Fredrickson et al., 1993). Improved pastures are commonly grazed by cattle in the southern United States where beef cattle production systems often take advantage of *Bos indicus* genetics. *Bos indicus* cattle are able to better tolerate high ambient temperatures, humidity, intense sunshine, and are able to resist parasites (Turner, 1980). *Bos taurus* cattle, conversely, evolved in cooler, more temperate climates (Howes et al., 1963) and are not as well adapted to the climatic conditions prevalent in the southern U.S. These cattle, however, have the advantage of providing superior quality carcasses and being early maturing (Forbes et al., 1998). These two subspecies of cattle are known to have differences in intake, digestion and ruminal fermentation parameters which are diet dependent (Howes et al., 1963; Frisch and Vercoe, 1969, 1977; Hunter and Siebert, 1985a,b; Habib et al., 2008).

Although the capacity of monensin to improve feed efficiency in cattle is widely recognized (Oliver, 1975; Raun et al., 1976; Thornton and Owens, 1981; Ward et al., 1990a), subspecies differences in response to monensin have not been evaluated. Based on the extent of digestive function differences between the subspecies, it seems appropriate to explore the effect of monensin on these parameters. Accordingly, this study was designed to evaluate subspecies differences in intake, digestion, and ruminal fermentation parameters in steers consuming bermudagrass hay, with or without monensin.

Materials and methods

Experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University, and included the use of anesthesia when surgical procedures were performed.

Animals

Ten steers (5 each: Bt, 400 ± 22 kg initial BW and Bi, 396 ± 33 kg initial BW) were fitted with ruminal and proximal duodenal (double- L shaped; Streeter et al., 1991) cannulas and assigned to concurrent two period, two treatment crossover designs by subspecies. During the diet adaptation period, steers were group housed in pens (29.0 m \times 50.3 m) with access to partially covered, individual pens (6.1 m \times 12.2 m). To facilitate sample collection, steers were confined to the individual pens d 18-25 and 41-42.

Diet and treatments

Animals had continuous access to fresh water and commercial trace mineral-salt blocks ($\geq 96\%$ NaCl, 1.00% S, 0.15% Fe, 0.25% Zn, 0.30% Mn, 0.009% I, 0.015% Cu, 0.0025% Co, and 0.001% Se; United Salt Corporation, Houston, TX). *Ad libitum* access to bermudagrass hay (*Cynodon dactylon*; Table D1) was provided via round bales while group housed. While individually housed, chopped (76 mm \times 76 mm wire mesh screen) hay was offered at 0800 daily at 130% of the previous 3 d average consumption, determined d 18-20. Treatments consisted of 0 or 200 mg \cdot hd⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN) fed daily in 0.91 kg DDGS at 0800 in individual pens. In period one, three steers from each subspecies were randomly

assigned to MON (200 mg·hd⁻¹·d⁻¹ monensin) and two to CON (0 mg·hd⁻¹·d⁻¹ monensin) treatment groups. By design, the second period comprised 3 CON and 2 MON steers within each subspecies.

Experimental protocol and sampling

The study consisted of two 70 d periods. Each period consisted of a 42 d treatment application period; 20 d adaptation followed by 22 d sample collection. By original design, the 22 d sample collection period was scheduled to encompass only 6 d (5 d for measures of intake and digestion and 1 d for ruminal fermentation parameters). However, collection of ruminal fluid was delayed for 17 d in the first period because of an unanticipated issue with the laboratory availability. Thus, the sample collection period was prolonged in both periods to accommodate this delay.

Following the 42 d treatment application period, 28 d were allowed for withdrawal of treatment prior to the second period, to prevent carry-over effects. During the 28 d withdrawal period, measures were taken for intake, digestion, and ruminal fermentation parameters to determine effects of monensin withdrawal. Results of the withdrawal period are reported in a subsequent paper.

Measures of intake and digestion occurred d 21-25. Hay samples were collected d 21-24 and composited within each period to correspond with ort and fecal samples collected d 22-25. Orts were collected immediately prior to the daily feeding on d 22-25 and composited within animal for each period. Fecal grab samples were collected three times daily beginning at 0800 on d 22. Fecal collection continued every 8 h for 4 d, with initial sampling time being delayed 2 h each day. Fecal samples were composited within

animal and frozen at -20°C for subsequent analysis. Distiller's dried grains with solubles samples were collected weekly throughout the study and composited within treatment for each period.

Rumen fluid was collected with a suction-strainer (Raun and Burroughs, 1962; 19 mm diameter, 1.5 mm mesh) from three locations within the dorsal and ventral sacs of the rumen 0, 2, 4, 8, and 12 h after feeding on d 42 for analysis of pH, VFA, and RAN. A portable pH meter (Symphony, VWR, Radnor, PA) was used to measure pH of rumen fluid at time of sampling. Subsequently, an 8 mL subsample of ruminal fluid was combined with 2 mL of freshly prepared 25% (wt/vol) metaphosphoric acid, then frozen at -20°C for later determination of VFA. An additional 9 mL subsample of ruminal fluid was combined with 1 mL of 1 N HCl, then frozen at -20°C for later determination of RAN. A separate ruminal fluid sample was collected 2 h after feeding on d 42 to determine rate of ruminal NH_3 production and CH_4 -producing activity. This sample was obtained by removing ruminal contents from three locations within the dorsal and ventral sacs of the rumen and squeezed through four layers of cheesecloth into insulated containers. Containers were filled, then capped to reduce oxygen exposure and immediately transported to the laboratory. In the laboratory, ruminal fluid was blended using an IKA Lab Egg (Wilmington, NC) and aliquots were apportioned for analyses of rate of ruminal NH_3 production and CH_4 -producing activity.

Laboratory analyses

Intake and digestion

Hay, ort and fecal samples were dried in a forced-air oven (96 h, 55°C) and allowed to air equilibrate to determine partial DM. Samples were ground (No. 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) to pass through a 1-mm screen. Hay, ort, supplement, and fecal samples were dried at 105°C for DM determination, then combusted for 8 h at 450°C for OM determination. Crude protein was determined by analyzing samples for N with an Elementar Rapid N Cube (Elementar, Hanua, Germany). Crude protein was calculated as $N \times 6.25$. Analyses for NDF and ADF were performed using an ANKOM fiber analyzer (ANKOM Technology, Macedon, NY) with sodium sulfite and α -amylase omitted. Acid detergent insoluble ash (ADIA) was used as an internal marker and determined on hay, ort, supplement, and fecal samples by combusting ANKOM bags containing ADF residue for 8 h at 450°C in a muffle furnace. Fecal production was estimated using ADIA intake divided by fecal ADIA concentration. Total tract digestion coefficients were calculated for OM and NDF using procedures as described by Cochran and Galyean (1994).

Volatile fatty acids

Ruminal fluid samples, previously prepared for VFA determination, were thawed and centrifuged at $15,000 \times g$ for 10 minutes at 22°C and supernatant fluid was collected and stored at -20°C for VFA analysis. Volatile fatty acid concentrations were measured, after thawing, using a gas chromatograph as described by (VanZant and Cochran, 1994).

Ruminal ammonia-N

Ruminal fluid samples, previously prepared for RAN determination, were thawed and centrifuged at $18,000 \times g$ for 10 min at 22°C. Ammonia was assayed colorimetrically using Berthelot's reaction according to Chaney and Marbach (1962). Concentrations were obtained using a Tecan Infinite M200 Pro Micro Plate Reader and Tecan Megellan Software (Tecan, Salzburg, Austria).

Rate of in vitro ruminal ammonia production

Rate of NH_3 -production was determined by incubation of 2 mL freshly collected rumen fluid with 6 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 2% (wt/vol) Bacto Trypticase Peptone (Becton, Dickinson and Company, Sparks, MD). Duplicate 18×150 mm crimp top culture tubes flushed with 100% CO_2 were sealed using rubber stoppers with aluminum crimps and incubated at 39°C. Immediately prior to (h 0) and 6 h after incubation a 0.5 mL sample was removed, then frozen for later analysis.

For analysis, frozen samples were thawed and centrifuged at $18,000 \times g$ for 10 min at 22°C. Ammonia was assayed colorimetrically using Berthelot's reaction according to Chaney and Marbach (1962). Rates of NH_3 production were calculated by dividing the difference between 6 and 0 h concentrations by 6 h incubation time; concentrations were obtained using a Tecan Infinite M200 Pro Micro Plate Reader and Tecan Megellan Software (Tecan, Salzburg, Austria).

In vitro ruminal CH₄-producing activity

In vitro ruminal CH₄-producing activity was determined by incubation of 5 mL freshly collected rumen fluid with 5 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 60mM sodium formate and 0.2g finely ground alfalfa as described by Anderson et al. (2006). Duplicate 18 × 150 mm crimp top culture tubes flushed with 50% H₂- 50% CO₂ were sealed using rubber stoppers and aluminum crimps and incubated 3h at 39°C. Upon conclusion of incubation period, a 1.0 mL gas sample was removed from the headspace of each tube and analyzed for CH₄ by gas chromatography on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ) equipped with a HaySep Q column (60°C, 25 mL/min of Argon carrier gas).

Statistical analysis

Intake, digestion, rate of ruminal NH₃ production and CH₄-producing activity were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included monensin, subspecies, monensin × subspecies and period with animal as a random effect. Ruminal fermentation parameters were analyzed using the MIXED procedure. Terms in the model included monensin, subspecies, hour, all their interactions, and period with animal × monensin × period and animal included as random effects. The repeated term was hour with animal × monensin as the subject. Treatment means were calculated using the LSMEANS option. The pdiff function was used to separate treatment means.

Results

No subspecies \times monensin interactions were observed for any intake or digestibility parameter measured. Monensin tended to increase forage OM intake (FOMI; $P = 0.08$; Table D2) from 19.5 to 20.3 g/kg BW and total OM intake (TOMI; $P = 0.07$) from 21.3 to 22.2 g/kg BW. No effect of subspecies ($P \geq 0.16$) was observed for FOMI or TOMI. Organic matter digestibility (OMD) was not affected ($P = 0.97$) by monensin (Table D3). When the combined effects of TOMI and OMD were evaluated as total digestible OM intake (TDOMI), no response ($P = 0.71$) was observed from monensin supplementation. *Bos taurus* steers had greater ($P = 0.04$; 14.3 vs 12.2 g/kg BW) TDOMI than *Bi* steers. Similarly, monensin tended to increase ($P \leq 0.08$) forage NDF intake (FNDFI; from 16.5 to 17.2) and total NDF intake (TNDFI; from 17.4 to 18.1 g/kg BW). Monensin did not alter ($P \geq 0.73$) NDF digestibility (NDFD) or total digestible NDF intake (TDNDFI). No effects of subspecies ($P \geq 0.16$) were observed for FOMI, FNDFI, TOMI, or TNDFI. Although OMD and NDFD were not different ($P \geq 0.22$) between subspecies (58.0 vs 63.3% OMD and 63.7 vs 68.7% NDFD, for *Bi* vs *Bt*, respectively), *Bt* steers had greater TDOMI (14.3 vs 12.2 g/kg BW) and TDNDFI (12.7 vs 10.9 g/kg BW) than *Bi* steers.

No subspecies \times monensin, subspecies \times time after feeding, or monensin \times time after feeding interactions were observed any ruminal fermentation parameter measured. There was a tendency ($P = 0.09$) for a subspecies \times monensin \times hour after feeding interaction for butyrate; however, this interaction was not significant ($P \geq 0.22$) for any other fermentation response.

Total VFA concentration was greater ($P = 0.01$) in CON vs MON steers (66.5 vs 62.0 mM; Table D4). Total VFA concentration was similar ($P = 0.28$) between subspecies (62.7 and 65.8 in Bi and Bt, respectively). There was an effect ($P < 0.01$) of hour after feeding on total VFA, with a steady decrease in total VFA immediately after feeding followed by an increase beginning 8 h after feeding (Fig D1).

The acetate:propionate ratio and molar percentages of acetate and propionate were not affected ($P \geq 0.18$) by subspecies. Monensin decreased the molar percentage of acetate ($P = 0.02$) from 72.5% to 71.2% and increased the molar percentage of propionate ($P < 0.01$) from 16.9% to 18.7%, reducing ($P < 0.01$) the acetate:propionate ratio from 4.34 to 3.85. There was an effect of hour after feeding ($P < 0.01$) on the acetate:propionate ratio and molar percentages of acetate and propionate. Immediately after feeding, molar percentage of acetate decreased (Fig. D2) and propionate increased (Fig. D3). However, 2 h after feeding molar percentage of acetate began to increase while propionate decreased. The acetate:propionate ratio followed a similar pattern to acetate (Fig. D4).

A tendency for subspecies \times monensin \times hour after feeding interaction ($P = 0.09$) was observed for butyrate, largely due to a greater molar percentage of butyrate in CON Bi steers at feeding and 2 h later, followed by a reduced concentration 4, 8, and 12 h after feeding (Fig. D5).

Molar percentages of isobutyrate, valerate, and isovalerate were similar between subspecies ($P \geq 0.12$) and treatment ($P \geq 0.14$). An effect of hour after feeding was observed ($P < 0.01$) for isobutyrate (Fig. D6), valerate (Fig. D7) and isovalerate (Fig.

D8). A reduction in molar percentage of isobutyrate and isovalerate were observed after feeding through h 12. Molar percentage of valerate increased until 4 h after feeding, then decreased 8 h and increased 12 h after feeding.

Ruminal NH₃-N was similar for CON and MON ($P = 0.72$; 1.55 vs. 1.57 mM). A subspecies \times hour after feeding interaction was observed for RAN (Fig. D9). *Bos taurus* steers had greater RAN than Bi at h 0 and 4; however, Bi had greater RAN than Bt 2, 8, and 12 h after feeding.

Rate of ruminal NH₃ production was similar between subspecies ($P = 0.11$; 0.05 and 0.09 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ for Bi and Bt, respectively) and treatment groups ($P = 0.33$; 0.06 vs 0.08 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ for CON vs MON, respectively; Table D5).

Monensin had no effect ($P = 0.19$) on CH₄-producing activity (20.6 vs 17.4 $\mu\text{mol CH}_4\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ for CON vs MON, respectively). *Bos indicus* steers tended to have greater ($P = 0.07$) CH₄-producing activity than Bt steers (21.37 vs 16.62 $\mu\text{mol CH}_4\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$).

Ruminal pH was not different between subspecies ($P = 0.55$; 6.67 and 6.62 for Bi and Bt, respectively) or treatment groups ($P = 0.35$; 6.62 vs 6.68 for CON vs MON, respectively). There was an effect of hour after feeding on ruminal pH with a reduction observed immediately after feeding, followed by an increase beginning 2 h after feeding and decline beginning 4 h after feeding (Fig. D10).

Discussion

Monensin is added to ruminant diets to improve animal production efficiency and mitigate enteric CH₄ production. By targeting and killing gram-positive bacteria,

including H₂, NH₃, and lactate-producing species, monensin alters ruminal fermentation (Dennis et al., 1981; Dinius et al., 1976; Van Nevel and DeMeyer, 1977). Comparisons of Bi and Bt reveal the difference in their ruminal fermentation parameters to be diet dependent (Hunter and Siebert, 1987; Hennessy et al., 2000). Thus, Brahman cattle were used to represent the Bi subspecies, while Angus cattle were used to represent the Bt subspecies to evaluate their differences in response to monensin while consuming bermudagrass hay. Effects of monensin and subspecies are discussed separately because no subspecies × monensin interactions were observed.

Effects of monensin inclusion

Intake

Monensin has variable effects on intake of cattle consuming forage-based diets. While the majority of studies report no monensin effects on forage intake (Oliver, 1975; Crosthwait et al., 1979; Ward et al., 1990a; Fredrickson et al., 1993; Vagnoni et al., 1995; Reed and Whisnant, 2001), a few report a reduction (Turner et al., 1977; Lemenager et al., 1978a,b; Ellis et al., 1983), and even fewer, an increase (DeLaney, 1980; Ellis et al., 1983). In a summary of 13 grazing trials, Ellis et al., (1983) suggested that the direction and magnitude of intake response to monensin is related to forage digestibility, the animal's physical capacity to harbor undigested residues, and their energy requirements. Specifically, it was suggested that monensin decreases intake of poor-quality (< 45% OMD) and high-quality (> 65% OMD) forages and increases intake of medium-quality forages (45 - 65% OMD); these suggestions were made using predominantly stocker cattle grazing wheat pasture. Organic matter digestibility of the

bermudagrass fed in this study averaged 62%; medium-quality according to the classification of Ellis et al. (1983). As Ellis et al. (1983) suggested, monensin increased FOMI by 4.4% and FNDFI by 4.4%. Similarly, DeLaney (1980) reported an increase, although larger, in OMI (21%) for heifers grazing a 13 hectare common bermudagrass pasture (9.0% CP; 49% OMD; medium quality according to the classification of Ellis et al., 1983) supplemented with 454 g cracked corn (80%) and cottonseed meal (20%) with 0 or 200 mg·hd⁻¹·d⁻¹ monensin. In a subsequent trial with the same animals, treatments, and medium quality diet (13.8% CP; 60.1% OMD), DeLaney (1980) again observed an increase in OMI (15.1%).

Digestion

Ellis et al. (1983) reported an average 4% increase in OMD in cattle receiving monensin, above that of controls, with the greatest effect occurring when the least digestible forages were fed. Several (Lemenager et al., 1978b; Pond and Ellis, 1979; Pond et al., 1980) have reported monensin to decrease rate of passage with high-fiber diets. Ellis et al. (1983) suggested that the decreased rate of passage with monensin may explain the increase in digestibility. In accordance with Ellis et al. (1983), Ward et al. (1990a) reported a 3% increase in OMD ($P < 0.05$) with monensin (101 mg·hd⁻¹·d⁻¹ via ruminal delivery device). In contrast to these studies, monensin had no effect on OMD in the present study. Similarly, Cochran et al. (1990) observed similar digestibilities (68.7 and 71.4% OMD) in cattle grazing early-summer bluestem range (12% CP) with or without a monensin (100 mg·hd⁻¹·d⁻¹ via ruminal delivery device). Furthermore, Ward et

al. (1990b) reported forage OMD to be similar (57.3 vs 57.5%) for control and monensin (101 mg·hd⁻¹·d⁻¹ via ruminal delivery device) steers grazing winter range (6.6% CP).

Volatile fatty acids

Relative amounts of VFA produced in the rumen are of particular interest because of their role in metabolic pathways in other organs. Propionate, a substrate for gluconeogenesis, is the main source of glucose for a ruminant animal. Acetate and butyrate, however, are non-gluconogenic, rather, they are precursors for long-chain fatty acid synthesis. Additionally, propionate is a hydrogen sink, whereas acetate and butyrate are hydrogen sources; hydrogen is a major substrate for CH₄ formation (Wolin, 1960). Therefore, the non-gluconogenic to gluconogenic VFA ratio is of importance because of its association with energy balance (Thomas and Martin, 1988).

Total VFA concentrations decreased steadily until 8 h after feeding, at which time concentrations escalated. Monensin reduced total VFA concentration by 6.8%. Similarly, Crosthwait et al. (1979) reported lower (4.0%) total VFA concentrations in ruminal fluid of heifers fed monensin (200 mg·hd⁻¹·d⁻¹) and Vagnoni et al. (1995) observed a more significant reduction (20.6%) in monensin (74 ppm) supplemented steers.

Molar proportion of acetate was reduced (by 1.74%) and propionate increased (by 10.43%) by monensin, as expected and in agreement with previous work (Dinius et al., 1976; Lemenager et al., 1978b; Vagnoni et al., 1995). Lemenager et al. (1978b) observed decreased molar proportion of acetate (24 h average: 72.6 vs 64.5) and increased propionate (24 h average: 21.2 vs 29.3) 4, 10, 16, and 22 h after feeding when a 30%

protein soybean meal supplement was fed to control vs monensin ($200 \text{ mg}\cdot\text{hd}^{-1}\cdot\text{d}^{-1}$) cows consuming harvested dry winter range grass. Similarly, Vagnoni et al. (1995) observed decreased proportions of acetate (73.1 vs 69.8) and increased proportions of propionate (17.5 vs 21.1) for control vs monensin ($200 \text{ mg}\cdot\text{hd}^{-1}\cdot\text{d}^{-1}$) steers consuming bermudagrass hay (5.3% CP). Consequently, the acetate:propionate ratio was reduced by monensin as it was with Lemenager et al. (1978b; from 3.4 to 2.2) and Vagnoni et al. (1995; from 4.2 vs 3.3).

Immediately after feeding, molar proportion of acetate declined and propionate increased. Two hours later, molar proportion of acetate began to increase while propionate decreased. By 8 h after feeding, molar proportion of acetate began to slowly decrease while propionate slowly increased. The acetate:propionate ratio followed a similar pattern to acetate.

Ruminal ammonia nitrogen

In accordance with previous work with monensin fed to cattle consuming forage based diets (Ward et al., 1990a; Fredrickson et al., 1993), monensin did not affect RAN. Ward et al. (1990a) observed RAN to be similar (7.1 vs 6.8 mM) between control and monensin treatments. Likewise, RAN concentrations were not affected (1.5 vs 1.6 mM for control and monensin, respectively) by ruminally dispensed monensin ($68 \text{ mg}\cdot\text{hd}^{-1}\cdot\text{d}^{-1}$ via ruminal delivery device) in a study by Fredrickson et al. (1993). Monensin sometimes decreases RAN concentrations (Poos et al., 1979; Chalupa, 1980; Branine, 1987) suggesting decreased protein degradation; however, if dietary protein degradation was altered in MON steers it was not reflected in RAN concentration. As could be

expected with a lack of treatment effect on RAN, rate of ruminal NH₃ production was also similar between treatments.

Methane-producing activity

Monensin had no effect on CH₄-producing activity. Zinn et al. (1994) also observed no change in CH₄ production in feedlot cattle consuming a steam-flaked corn-based finishing diet (12.1% CP) with monensin (209 mg·hd⁻¹·d⁻¹). Short term decreases in CH₄ production have been reported (up to 30%), however, the inhibitory effects of monensin may not persist over time (Rumpler et al., 1986; Guan et al., 2006). Rumpler et al. (1986) reported CH₄ production returned to control values by d 12 of monensin (226 mg·hd⁻¹·d⁻¹) supplementation in beef steers consuming a 70% cracked corn basal diet. Additionally, CH₄ production in a study by Guan et al. (2006) returned to baseline level by the third week of monensin inclusion when cattle were fed a high forage diet (13.1% CP) and by the sixth week of monensin inclusion when fed a high concentrate diet (13.8% CP). Thus, if monensin had any effect on CH₄ production in the current study, in accordance with previous work (Rumpler et al., 1986; Guan et al., 2006), it did not persist 42 d after commencing monensin inclusion, when CH₄-producing activity was measured.

Effects of subspecies

Intake

Bos taurus cattle are generally observed to have greater intakes than Bi when consuming forages of various quality (Ledger et al., 1970; Frisch and Vercoe, 1977; Hennessy et al., 2000; Habib et al., 2008). Hunter and Siebert (1986) observed 20%

greater consumption (27.6 vs 23.0 g DM/kg BW) in Bt than Bi consuming high quality forage (lucerne; 18.44% CP). However, when these cattle consumed a lower quality forage (speargrass; 3.88% CP) similar intakes (11.3 and 11.8 g DM/kg BW) were observed in Bt than Bi. Similarly, in a forage diet comprising highly digestible (69% OMD) German grass (*Echinochloa crusgalli*; 6.10% CP) and less digestible (58% OMD) rice straw (4.8% CP) Habib et al. (2008) observed an 8.0% greater voluntary DM intake (14.21 vs 12.87 g/kg BW) for consumption of German grass by Bt×Bi cross vs Bi steers. However, consumption of rice straw was not different between subspecies (9.44 vs 9.21 g/kg BW, Bt×Bi vs Bi, respectively). Therefore, the difference between subspecies appears to be diet dependent, with them becoming more similar when lower quality, N-deficient forages are fed (Hunter and Siebert, 1986, 1987; Habib et al., 2008). Although the bermudagrass forage fed in the present study was not low-quality or N-deficient (13.2% CP; 62% OMD), a subspecies difference was not observed for FOMI or FNDFI. This observation is not unprecedented as it is similar to Habib et al. (2011). In a trial investigating the effects of four various quality diets daily DM intake and forage intake were not different ($P > 0.05$; 27.85, 28.84, 30.06, and 29.70 g/kg BW, for 11.7, 7.6, 7.3, and 3.7% CP diets, respectively) for Bi and Bi×Bt steers (Habib et al., 2011).

Digestion

Differences in fiber digestion of Bi and Bt were first investigated by French (1940). Although French (1940) did not observe significant differences (68.1 vs 60.1% for Bi and Bt respectively), he was the first to suggest greater fiber digestion in Bi than Bt consuming hay in a series of 10 trials. Hungate et al. (1960) and Phillips et al. (1960)

followed and observed that Bi had 8.1 (182.2 vs 168.5 $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$; Hungate et al., 1960) and 17.9% (170.7 vs 144.8 $\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$; Phillips et al., 1960) faster rates of fermentation than Bt when low-quality forages were fed; although neither were able to demonstrate significant differences, possibly due to the number of animals used. A number of studies indicate that Bi cattle may have greater ability to digest low-quality forages than Bt (French, 1940; Phillips et al., 1960; Habib et al., 2008); however, these differences are small. Habib et al. (2011) was able to observe significant differences, with Bi having greater OMD than Bt steers consuming rice straw (61.2 vs 54.7%), but similar OMD when they consumed German grass (68.5 vs 68.5%). In accordance with Habib et al. (2011), the 62.0% OMD bermudagrass hay fed during this trial, did not result in subspecies differences in OMD or NDFD. *Bos taurus* steers had greater TDOMI than Bi resulting from numerically greater OMD (9.2%) and TOMI (6.5%) for Bt vs Bi steers. Similarly, numerically greater NDFD (7.8%) and TNDFI (6.8%) resulted in greater (16.3%) TDNDFI in Bt than Bi steers.

Volatile fatty acids

Limited data is available (Weldon, 2013) concerning subspecies differences in total VFA concentration or molar proportions of acetate or propionate. Weldon (2013) observed no subspecies difference in total VFA concentration or the acetate:propionate ratio. Similarly, in the present study, total VFA concentration and the acetate:propionate ratio were similar for Bi and Bt steers.

Ruminal ammonia nitrogen

Hunter and Siebert (1985a) observed Bi to have greater RAN concentrations on low-N diets than Bt. Similarly, Bi steers had greater RAN concentrations than either Bt or Bi × Bt crosses when consuming low-quality hay (4.3% CP; Hennessy et al., 2000).

However, as the dietary N supply increases, the subspecies difference in NH₃ becomes insignificant (Hunter and Siebert, 1985a). Although the bermudagrass diet was not N-deficient, Bi steers were observed with greater RAN than Bt 2, 8, and 12 h after feeding; however, Bt had greater RAN than Bi immediately prior to feeding (h 0) and 4 h later. Although subspecies interacted with hour after feeding concerning RAN concentration, no effect of subspecies, hour after feeding, or their interaction was observed for rate of ruminal NH₃ production.

Methane-producing activity

The association of methanogenic archaea with rumen ciliates is a common feature of anaerobic systems (Vogels et al., 1980). Ciliate protozoa depend on a hydrogen evolving fermentation, however, they benefit from hydrogen removal because of its inhibitory effect on their metabolism if not removed (Sharp et al., 1998). Methanogens attached to protozoa derive H₂ directly from specialized organs called hydrogenosomes (Hungate, 1966). The attachment is symbiotic in that it provides for interspecies hydrogen transfer. *Bos indicus* cattle frequently have greater numbers of rumen protozoa (Hennessy et al., 1995), hence it is reasonable to assume that they would support greater CH₄ emissions. Accordingly, Bi steers in the present study were observed to have greater *in vitro* CH₄-producing activity than Bt.

CHAPTER VI

EFFECT OF MONENSIN WITHDRAWAL ON INTAKE, DIGESTION, AND RUMINAL FERMENTATION PARAMETERS IN *BOS INDICUS* AND *BOS TAURUS* STEERS CONSUMING BERMUDAGRASS HAY

Overview

Effects of monensin withdrawal and cattle subspecies on the utilization of bermudagrass hay (13.7% CP) were evaluated using ruminally cannulated steers (5 *Bos indicus*, Bi and 5 *Bos taurus*, Bt; 398 kg BW). Subspecies were concurrently subjected to a two period, two treatment crossover design. Treatments consisted of withdrawal from either 0 (CON) or 200 (MON) mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN) fed individually in 0.91 kg DDGS, for 42 d. Withdrawal was evaluated for a 28 d period. Ruminal fluid was collected 2 h after feeding on d 1, 4, 7, 14, and 21 post withdrawal for determination of pH, VFA, ruminal NH₃-N (RAN), rate of NH₃ production and CH₄-producing activity. Hay, ort, and fecal grab samples were collected 23-27 d post withdrawal for determination of intake and digestion. No subspecies × monensin, subspecies × d, or subspecies × monensin × d interactions were observed ($P \geq 0.11$). An effect of d was observed ($P < 0.01$) for total VFA concentration, with an increase following withdrawal, followed by a decrease then stabilization. Monensin × day interactions ($P \leq 0.01$) were observed for the acetate:propionate ratio and molar % of acetate and propionate. There was a decrease in molar % propionate between d 1 and 4 from 19.1 to 18.0%; however, it remained greater ($P \leq 0.10$) for MON

than CON through d 7. Monensin withdrawal increased molar % acetate from 68.3 to 69.8 between d 0 and 4 for MON steers. Acetate:propionate ratio was lower ($P \leq 0.01$) on d 0 for MON than CON (3.4 vs 4.0), but by d 4, it increased to 3.8, and was not different ($P = 0.14$) from CON. By d 14, no differences ($P \geq 0.88$) remained for acetate, propionate, or acetate:propionate ratio. Greater RAN ($P < 0.01$; 2.09 vs 1.83 mM) was observed in CON than MON steers. Monensin had no effect on rate of NH_3 production or CH_4 -producing activity. Greater forage OM intake (FOMI; $P = 0.09$; 21.2 vs 19.2 g/kg BW) and OM digestibility (OMD; $P < 0.01$; 72.4% vs 63.0%) resulted in greater ($P < 0.01$) total digestible OM intake (TDOMI; 16.8 vs 13.2 g/kg BW) in Bt vs Bi steers. Previous monensin feeding had no effect ($P = 0.45$) on OMD (66.8 and 68.6 for CON and MON, respectively). Increased FOMI, OMD and TDOMI by Bt steers suggest they are better able to utilize the bermudagrass hay offered them than Bi steers. Results also indicate that upon withdrawal, steers previously fed monensin continue to have reduced acetate:propionate ratio for at least 7 d.

Introduction

Monensin is added to ruminant rations to improve feed utilization, prevent acidosis, and mitigate enteric CH_4 production. Although its effectiveness has been documented in grazing systems (Oliver, 1975; Lemeneger et al., 1978a; Crosthwait et al., 1979; Rouquette et al., 1980; Ellis et al., 1983; Ward et al., 1990a; Fredrickson et al., 1993), difficulty associated with providing monensin to grazing cattle has prevented its widespread use. Some producers, however, take advantage of monensin during critical

periods, such as late pregnancy or peak lactation, allowing them to obtain its benefits, while only enduring the cost of supplementation for short periods of time.

A dearth of information exists (Lemeneger et al., 1976; Dawson and Boling, 1983; Rogers et al., 1997) concerning the lasting effects of monensin upon its withdrawal. This information could aid producers in determining optimal time to feed monensin to obtain a desired response and create a more economically efficient production system.

Furthermore, in the southern United States, beef cattle production systems often take advantage of *Bos indicus* influenced cattle. *Bos indicus* cattle are able to tolerate the high ambient temperatures, humidity, and intense sunshine (Turner, 1980) prevalent in this area. *Bos taurus* cattle, however, are not as well adapted, having evolved in cooler, more temperate climates (Howes et al., 1963). Based on the extent of digestive function differences between the two subspecies (Howes et al., 1963; Hunter and Siebert, 1985a; Hennessy et al., 1995, 2000), and potential benefits of intermittently feeding monensin to cattle in grazing systems, our objectives were to explore 1) the effect of monensin withdrawal on intake, digestion, and ruminal fermentation parameters 2) differences in response to monensin withdrawal by Bi and Bt subspecies of cattle and 3) the potential interactions of subspecies with monensin.

Materials and methods

Experimental protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University, and included the use of anesthesia when surgical procedures were performed.

Animals

Ten steers (5 each: Bt, 400 ± 22 kg initial BW and Bi, 396 ± 33 kg initial BW) were fitted with ruminal and proximal duodenal (double- L shaped; Streeter et al., 1991) cannulas and assigned to concurrent two period, two treatment crossover designs by subspecies. During the diet adaptation period, steers were group housed in pens (29.0 m \times 50.3 m) with access to partially covered, individual pens (6.1 m \times 12.2 m). To facilitate sample collection, steers were confined to the individual pens d 0, 1, 4, 7, 14 and 20-28.

Diet and treatments

Animals had continuous access to fresh water and commercial trace mineral-salt blocks ($\geq 96\%$ NaCl, 1.00% S, 0.15% Fe, 0.25% Zn, 0.30% Mn, 0.009% I, 0.015% Cu, 0.0025% Co, and 0.001% Se; United Salt Corporation, Houston, TX). *Ad libitum* access to bermudagrass (*Cynodon dactylon*) hay (Table E1) was provided via round bales while group housed. While individually housed, chopped (76 mm \times 76 mm wire mesh screen) hay was offered at 0800 daily at 130% of the previous 3 d average consumption, determined d 20-22 after monensin withdrawal. Treatments consisted of 0 or 200 mg \cdot hd⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN) fed daily in 0.91 kg DDGS at 0800 in individual pens. Steers within each subspecies were randomly assigned to treatment groups. In the first period, three steers were randomly assigned to the MON (200 mg \cdot hd⁻¹ \cdot d⁻¹ monensin) and two to the CON (0 mg \cdot hd⁻¹ \cdot d⁻¹ monensin) treatment group. By design, the second period comprised 3 CON and 2 MON steers within each subspecies.

Experimental protocol and sampling

The study comprised two periods, each consisting of a 42 d treatment application phase followed by a 28 d phase in which monensin was withdrawn from DDGS. Effects of monensin inclusion are reported in Chapter V.

Measures of intake and digestion occurred d 23-27 after the cessation of monensin application. Hay samples were collected d 23-26 and composited within each period to correspond with ort and fecal samples collected d 24-27. Orts were collected immediately prior to the daily feeding on d 24-27 and composited within animal for each period. Fecal grab samples were collected three times daily beginning at 0800 on d 24. Fecal collection continued every 8 h for 4 d, with initial sampling time being delayed 2 h each day. Fecal samples were composited within animal and frozen at -20°C for subsequent analysis. Samples of distiller's dried grains with solubles were collected weekly throughout the study and composited within treatment for each period.

Rumen fluid was collected with a suction-strainer (Raun and Burroughs, 1962; 19 mm diameter, 1.5 mm mesh) from three locations within the dorsal and ventral sacs of the rumen 2 h after feeding on d 1, 4, 7, 14, 21, and 28 for analysis of pH, VFA, and RAN. A portable pH meter (Symphony, VWR, Radnor, PA) was used to measure pH of rumen fluid at time of sampling. Subsequently, an 8 mL subsample of ruminal fluid was combined with 2 mL of freshly prepared 25% (wt/vol) metaphosphoric acid, then frozen at -20°C for later determination of VFA. An additional 9 mL subsample of ruminal fluid was combined with 1 mL of 1 N HCl, then frozen at -20°C for later determination of RAN. Separate ruminal fluid samples were collected 2 h after feeding on d 1, 4, 7, 14,

21, and 28 to determine rate of ruminal NH₃ production and CH₄-producing activity. These samples were obtained by removing ruminal contents from three locations within the dorsal and ventral sacs of the rumen then squeezing through four layers of cheesecloth into insulated containers. Containers were filled, then capped to reduce oxygen exposure and immediately transported to the laboratory. In the laboratory, ruminal fluid was blended using an IKA Lab Egg (Wilmington, NC) and aliquots were apportioned for analyses of rate of ruminal NH₃-production and CH₄-producing activity.

Laboratory analyses

Volatile fatty acids

Ruminal fluid samples were thawed and centrifuged at 15,000 × *g* for 10 minutes at 22°C and supernatant fluid was collected and stored at -20°C for VFA analysis. Volatile fatty acid concentrations were measured, after thawing, using a gas chromatograph as described by (VanZant and Cochran, 1994).

In vitro ruminal CH₄-producing activity

In vitro ruminal CH₄-producing activity was determined by incubation of 5 mL freshly collected rumen fluid with 5 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 60mM sodium formate and 0.2g finely ground alfalfa as described by Anderson et al. (2006). Duplicate 18 × 150 mm crimp top culture tubes flushed with 50% H₂- 50% CO₂ were sealed using rubber stoppers and aluminum crimps and incubated 3 h at 39°C. Upon conclusion of incubation period, a 1.0 mL gas sample was removed from the headspace of each tube and analyzed for CH₄ by gas chromatography on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac

Instrument, Bridgewater, NJ) equipped with a HaySep Q column (60°C, 25 mL/min of Argon carrier gas).

Ruminal ammonia-N

Ruminal fluid samples were thawed and centrifuged at $18,000 \times g$ for 10 min at 22°C. Ammonia was assayed colorimetrically using Berthelot's reaction according to Chaney and Marbach (1962). Concentrations were obtained using a Tecan Infinite M200 Pro Micro Plate Reader and Tecan Megellan Software (Tecan, Salzburg, Austria).

Rate of in vitro ruminal ammonia production

Ammonia-producing activity was determined by incubation of 2 mL of freshly collected rumen fluid with 6 mL anaerobic dilution solution (Bryant and Burkey, 1953) containing 2% (wt/vol) Bacto Trypticase Peptone (Becton, Dickinson and Company, Sparks, MD). Duplicate 18×150 mm crimp top culture tubes flushed with 100% CO₂ were sealed using rubber stoppers with aluminum crimps and incubated at 39°C. Immediately prior to (h 0) and after 6 h incubation a 0.5 mL sample was removed then frozen for later analysis of rate of ruminal NH₃ production.

For analysis, frozen samples were thawed and centrifuged at $18,000 \times g$ for 10 min at 22°C. Ammonia was assayed colorimetrically using Berthelot's reaction according to Chaney and Marbach (1962). Rates of NH₃ production were calculated by dividing the difference between 6 and 0 h concentrations by 6 h incubation time; concentrations were obtained using a Tecan Infinite M200 Pro Micro Plate Reader and Tecan Megellan Software (Tecan, Salzburg, Austria).

Intake and digestion

Hay, ort and fecal samples were dried in a forced-air oven (96 h, 55°C) and allowed to air equilibrate to determine partial DM. Samples were ground (No. 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) to pass through a 1-mm screen. Hay, ort, supplement, and fecal samples were dried at 105°C for DM determination, then combusted for 8 h at 450°C for OM determination. Crude protein was determined by analyzing samples for N with an Elementar Rapid N Cube (Elementar, Hanua, Germany). Crude protein was calculated as $N \times 6.25$. Analyses for NDF and ADF were performed using an ANKOM fiber analyzer (ANKOM Technology, Macedon, NY) with sodium sulfite and α -amylase omitted. Acid detergent insoluble ash (ADIA) was used as an internal marker and determined on hay, orts, supplement, and fecal samples by combusting ANKOM bags containing ADF residue for 8 h at 450°C in a muffle furnace. Fecal production was estimated using ADIA intake divided by fecal ADIA concentration. Total tract digestion coefficients were calculated for OM and NDF using procedures as described by Cochran and Galyean (1994).

Statistical analysis

Intake and digestion were analyzed using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, NC). Terms in the model included monensin, subspecies, monensin \times subspecies and period with animal as a random effect. Ruminal fermentation parameters (VFA, RAN, pH, rate of ruminal NH_3 production, and CH_4 -producing activity) were analyzed using the MIXED procedure. Terms in the model included monensin, subspecies, day, all their interactions, and period with animal \times monensin \times period and

animal included as random effects. The repeated term was day with animal \times monensin as the subject. Treatment means were calculated using the LSMEANS option. The pdiff function was used to separate treatment means.

Results

There were no subspecies \times monensin \times day after withdrawal ($P \geq 0.18$), subspecies \times monensin ($P \geq 0.11$) or subspecies \times day after withdrawal ($P \geq 0.29$) interactions for any of the fermentation data.

A monensin \times day after withdrawal interaction or effects of subspecies or monensin were not observed ($P \geq 0.18$) for total VFA concentration (Table E2). An effect of day after withdrawal was observed ($P < 0.01$) with a decrease occurring immediately after withdrawal followed by an increase from d 4 to 7 and stable concentration (72.6 – 77.6, molar %) for the remainder of the withdrawal period (Fig. E1).

An effect of subspecies was not observed ($P \geq 0.20$) for acetate, propionate or acetate:propionate ratio. Monensin \times day after withdrawal interactions ($P \leq 0.01$) were observed for molar percentages of acetate (Fig. E2) and propionate (Fig. E3) and the acetate:propionate ratio (Fig. E4). Monensin withdrawal caused an increase in molar percentage of acetate in MON steers from 68.3 to 69.8% between d 0 and 4. Although the biological significance of this change is questionable, the molar percentage of acetate tended to remain lower for MON than CON through d 7 ($P = 0.06$). A decrease (from 19.9 to 18.3%) was observed in molar percentage of propionate in MON steers between d 1 and 4. Molar % propionate remained greater ($P \leq 0.05$) for MON than CON through

d 7. Acetate:propionate ratio was lower ($P \leq 0.01$) on d 0 for MON than CON (3.4 vs 4.0). By d 4, the acetate:propionate ratio for MON increased to 3.8, and was not different ($P = 0.14$) from CON. By d 14, no differences ($P \geq 0.88$) between MON and CON remained for acetate, propionate, and acetate:propionate ratio.

An effect of subspecies was not observed ($P = 0.30$) for butyrate. A monensin \times day interaction was observed ($P < 0.01$) for butyrate. Monensin reduced molar percentage of butyrate throughout the withdrawal period, with the exception of d 7 (Fig. E5).

An effect of subspecies was not observed ($P = 0.21$) for isobutyrate. A tendency for monensin \times day after withdrawal interaction was observed ($P = 0.10$) for isobutyrate. Similar to butyrate, the molar percentage of isobutyrate remained greater in CON than MON steers throughout the withdrawal period with the exception of d 7 (Fig. E6).

A monensin \times day after withdrawal interaction or effect of subspecies was not observed ($P \geq 0.19$) for valerate. Monensin tended to increase ($P = 0.06$) the molar percentage of valerate from 1.04 to 1.11%. A tendency for effect of day after withdrawal was observed ($P = 0.06$) for valerate with an increase occurring between d 0 and 4 followed by a decrease from d 4 to 21 (Fig. E7).

A monensin \times day after withdrawal interaction was not observed ($P = 0.27$) for isovalerate. Monensin increased ($P \geq 0.04$) molar percentage of isovalerate from 0.97 to 1.05%. *Bos taurus* were observed to have greater ($P = 0.02$) molar percentage of isovalerate than Bi (1.06 vs 0.97%) steers. An effect of day after withdrawal was observed for isovalerate with a decrease immediately after withdrawal followed by an increase then stabilization (1.01 - 1.09%) by d 4 (Fig. E8).

A monensin \times day after withdrawal interaction or effects of monensin or subspecies were not observed ($P \geq 0.23$) for CH₄-producing activity (Table E3). An effect of day after withdrawal was observed with CH₄-producing activity decreasing after withdrawal, then increasing d 1 to 21 from 16.5 to 25.5 $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ (Fig. E9).

A monensin \times day after withdrawal interaction or effect of subspecies was not observed ($P \geq 0.33$) for RAN. Steers previously fed monensin had reduced ($P < 0.01$) RAN throughout the withdrawal period (Fig. E10). An effect of day after withdrawal was observed ($P < 0.01$) with RAN concentration remaining stable (1.79 – 1.92 mM) after withdrawal until d 4, then increasing to 2.30 mM on d 7 and finally decreasing to 1.83 mM by d 21.

A monensin \times day interaction or effects of monensin or subspecies were not observed ($P \geq 0.23$) for rate of ruminal NH₃ production. An effect of day after monensin withdrawal was observed ($P < 0.01$) for rate of ruminal NH₃ production (Fig. E11). Rate of ruminal NH₃ production ($\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$) increased from d 0 to 1 (0.07 to 0.31) and 7 to 14 (0.22 to 0.38), and decreasing from d 1 to 7 (0.31 to 0.22) and 14 to 21 (0.38 to 0.26).

A monensin \times day after withdrawal interaction was observed ($P = 0.03$) for pH. Higher pH was observed in MON steers throughout the withdrawal period, with the exception of d 7 (Fig E12). *Bos indicus* steers tended to have a higher ($P = 0.08$) pH than Bt steers after withdrawal (6.53 vs 6.46).

A subspecies \times monensin interaction was not observed ($P \geq 0.15$) for intake or digestion. When monensin was withdrawn for 23-27 days, forage OM intake (FOMI),

total OM intake (TOMI), OM digestibility (OMD), and total digestible OM intake (TDOMI) were not different ($P \geq 0.45$) between steers previously receiving monensin and control steers (Table E4). There was a tendency for Bt steers to have greater ($P \leq 0.10$) FOMI (21.2 vs 19.2 g/kg BW) and TOMI (23.0 vs 21.0 g/kg BW) than Bi steers. Additionally, Bt steers had greater ($P < 0.01$) OMD (72.4 vs 63.0%; Table E5), thus, when the combined effects of TOMI and OMD were evaluated as TDOMI, greater TDOMI was observed ($P < 0.01$; 16.8 vs 13.2 g/kg BW) in Bt vs Bi steers.

Similarly, forage NDF intake (FNDFI), total NDF intake (TNDFI), NDF digestibility (NDFD) and total digestible NDF intake (TDNDFI) were not different ($P \geq 0.42$) between steers previously receiving monensin and control steers. Greater FNDFI ($P = 0.04$; 16.7 vs 14.9 g/kg BW) and TNDFI ($P = 0.05$; 17.5 vs 15.8 g/kg BW) combined with greater NDFD ($P < 0.01$; 73.8% vs 65.6%) resulted in greater TDNDFI ($P < 0.01$; 12.9 vs 10.3 g/kg BW) for Bt vs Bi steers.

Discussion

Grazing systems are generally not equipped to feed cattle on a daily basis, as is required by monensin feeding, so the provision of monensin becomes an economic burden to the producer through additional resource requirements (carrier feed, bunks, trucks, labor, etc). However, feeding monensin for a short period of time may allow the producer to realize its benefits without enduring a significant economic impact. Lasting effects of monensin, post withdrawal, have not been evaluated and could impact the optimal length of time for its feeding to obtain the desired response. Furthermore,

because of the prevalence of Bi influenced cattle in the southern United States, understanding the differences in effects of monensin withdrawal on intake, digestion, and ruminal fermentation parameters between the two subspecies could prove beneficial. Thus, Brahman cattle were used to represent the Bi subspecies, while Angus cattle were used to represent the Bt subspecies to evaluate differences in response to monensin withdrawal while consuming bermudagrass hay. Effects of monensin and subspecies are discussed separately because no subspecies \times monensin interactions were observed.

Effects of monensin inclusion

Volatile fatty acids

Dawson and Boling (1983) reported that total VFA concentrations in monensin fed heifers consuming 3 kg of a basal diet (20% (wt/wt) cottonseed hulls, 11% soybean meal, 68% cracked corn, and 1% trace mineral salts twice daily) with $99 \text{ mg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$ monensin returned to values comparable to control animals within 10 d after monensin withdrawal; samples were taken at approximately weekly intervals, thus the exact time at which total VFA concentration became similar between monensin treated and control steers is unknown. Two others (Lemeneger et al., 1976; Rogers et al., 1997) have evaluated the effects of monensin withdrawal on total VFA concentration. Rogers et al. (1997) observed a 21.8% reduction in total VFA concentration (from 113.9 to 89.1 mM) when monensin ($33 \text{ mg monensin} \cdot \text{kg}^{-1}$ total diet) was included in the diet (14.4% CP pelleted diet fed at $45 \text{ g} \cdot \text{kg}^{-1} \text{BW}^{0.75}$ together with wheat straw) of wethers for 146 d, but no difference within 24 h of its withdrawal (55.2 and 62.2 for CON and MON, respectively). Likewise, when sampled 22 h post monensin feeding, Lemeneger et al.

(1976) observed no difference in total VFA concentration (42.3 vs 40.0 %) in cows grazing pasture and supplemented with 1.36 kg·hd⁻¹·d⁻¹ range cubes (30% natural protein supplement) with 0 or 200 mg·hd⁻¹·d⁻¹ monensin.

Volatile fatty acids, in the present study, were measured d 0 (the final day of monensin inclusion), and d 1, 4, 7, 14, and 21 after monensin withdrawal. In general, differences between the CON and MON groups diminished over time. Monensin reduced total VFA concentration by 6.7% in the monensin inclusion phase of this trial (Chapter V). Upon withdrawal, total VFA concentration remained lower (13.9%) for 1 d (26 h) post withdrawal in the steers previously treated with monensin. By d 4 post withdrawal, total VFA concentration was similar between CON and MON (64.5 and 63.7, respectively) and remained similar for the duration of the trial.

When sampled 22 h post monensin feeding, Lemenager et al. (1976) observed no difference in molar percentage of acetate (73.4 vs 73.4 %) or propionate (20.4 vs 21.1 %) in steers fed 0 or 200 mg·hd⁻¹·d⁻¹ monensin. Rogers et al. (1997) observed a decrease in the acetate:propionate ratio (from 3.30 to 2.15) while monensin was included in the feed, however, when monensin was withdrawn (for 27 d), the acetate:propionate ratio was increased (from 2.97 to 3.77). In the present study, the acetate:propionate ratio was lower in the MON group through 7 d post-withdrawal (3.69 vs 3.89). By d 14, the acetate:propionate ratio was similar (3.84 vs 3.85 for CON and MON, respectively) between treatments. Positive benefits exerted by monensin on the acetate:propionate ratio persisted for at least one week after monensin withdrawal.

Literature is limited concerning the effect of monensin withdrawal on butyrate and the minor VFA. When sampled 22 h post monensin feeding, Lemenager et al. (1976) observed no difference in molar percentage of butyrate (6.16 vs 5.53 %) in steers fed 0 or 200 mg·hd⁻¹·d⁻¹ monensin. Differences between treatment groups for butyrate in the present study did not follow a clear pattern. Treatment differences were observed d 1 and 21 post-withdrawal with the MON steers having lower proportions of butyrate than CON. However, this was not a consistent difference, as MON steers had greater proportions of butyrate 7 d post-withdrawal.

Methane-producing activity

It has been suggested (Rumpler et al., 1986; Guan et al., 2006) that the inhibitory effects of monensin on CH₄ production do not persist over long periods of time. Rumpler et al. (1986) reported CH₄ production returned to control values by d 12 of monensin (226 mg·hd⁻¹·d⁻¹) supplementation in beef steers consuming a 70% cracked corn basal diet. Additionally, CH₄ production in a study by Guan et al. (2006) returned to baseline level by the third week of monensin inclusion when cattle were fed a high forage diet (13.1% CP) and by the sixth week of monensin inclusion when fed a high concentrate diet (13.8% CP). Having fed monensin for 42 d before withdrawal, with no treatment difference observed ($P > 0.10$; Chapter V), no effect on CH₄-producing activity was observed or expected at any time after withdrawal.

Ruminal ammonia nitrogen

Monensin decreased RAN concentration by 8.9%. Literature suggests that monensin often reduces RAN concentration (Poos et al., 1979; Chalupa, 1980; Branine, 1987;

Rogers et al., 1997), because of its inhibitory effects on NH₃-producing bacteria, which would suggest decreased protein degradation. However, previous work with monensin fed to cattle consuming forage based diets reveals that monensin does not always exert this effect on RAN (Ward et al., 1990a, Fredrickson et al., 1993). Ward et al. (1990a) found RAN to be similar (12.1 vs 11.5 mg/100 mL) between control and monensin treated animals. Similarly, in the monensin inclusion phase of this trial (Chapter V), RAN was similar for CON and MON steers (1.55 and 1.57 mM, respectively). Rogers et al. (1997) evaluated the effect of monensin withdrawal on RAN and observed a 17.6% decline in wethers receiving monensin, but this difference diminished upon monensin withdrawal and RAN was similar between treatment groups (181 and 234 µg·mL⁻¹ for CON and MON, respectively).

Rate of *in vitro* ruminal NH₃-production varied from 0.23 to 0.43 µmol·mL⁻¹·h⁻¹, increasing and decreasing continually throughout the withdrawal period. Although monensin increased RAN concentration, no effect of monensin was observed for rate of *in vitro* ruminal NH₃ production.

Intake

Monensin has variable effects on intake of cattle consuming forage based diets. Monensin generally does not exert an effect on intake of forage based diets (Oliver, 1975; Crosthwait et al., 1979; Ward et al., 1990a; Fredrickson et al., 1993; Vagnoni et al., 1995; Reed and Whisnant, 2001), however, few report a reduction (Turner et al., 1977; Lemenager et al., 1978a,b; Ellis et al., 1983), and even fewer, an increase (DeLaney, 1980; Ellis et al., 1983) in intake. Ellis et al., (1983) suggested, in a summary

of 13 grazing trials using primarily stocker cattle grazing wheat pasture, that the direction and magnitude of intake response to monensin is related to forage digestibility, the animal's physical capacity to harbor undigested residues, and their energy requirements. Specifically, it was suggested that monensin increases intake of medium-quality forages (45-65% OMD) and decreases intake of poor-quality (< 45% OMD) and high-quality (> 65% OMD) forages. Organic matter digestibility of the bermudagrass hay fed in the present study averaged 62%; medium-quality according to the classification of Ellis et al. (1983). Accordingly, monensin increased forage intake during the monensin application phase of this trial (Chapter V). This difference diminished by d 23-27 after withdrawal, when intake measurements were taken, and effects on intake (FOMI, TOMI, FNDFI, or TNDFI) were no longer observed. The exact time after withdrawal in which intake became similar between treatments remains unknown. Without further studies regarding the effect of monensin withdrawal on intake, it is difficult to interpret this observation. However, it is reasonable to assume that intake will correspond with the diminishing effect on the acetate:propionate ratio. Monensin kept the acetate:propionate ratio lower than CON for 7 d post-withdrawal, but it was similar between treatments by 14 d post withdrawal. By d 23-27 post-withdrawal, when intake measurements were taken, lasting effects of monensin on the acetate:propionate ratio had diminished. Consequently, intake differences were no longer evident, as they likely diminished with the increasing acetate:propionate ratio.

Digestion

Only one other (Rogers et al., 1997) has observed the effects of monensin withdrawal on digestion. Rogers et al. (1997), observed OMD to increase from 67.0 to 71.5 when 33 mg monensin·kg⁻¹ diet was included. However, no apparent effect of monensin on OMD (70.0 vs 67.3 % for MON vs CON, respectively) remained after 27 d of withdrawal.

Unlike Rogers et al. (1997), monensin had no effect on digestibility during the monensin inclusion phase of this trial (Chapter V). Accordingly, 23-27 d after withdrawal, when digestion was measured, no effect of monensin was observed on OMD or NDFD.

Effects of subspecies

Volatile fatty acids

Limited data (Weldon, 2013) exist concerning differences in VFA concentrations in Bi and Bt cattle. Weldon (2013) observed greater total VFA concentrations (75.85 vs 69.78 mM) in Bt than Bi steers consuming low-quality rice straw (4.6% CP) supplemented with 50 or 100 mg N/kg BW high DIP (72%), or 100 mg N/kg BW low DIP (28%) supplement. In contrast, a subspecies difference was not observed in the present study for total VFA, however, this observation is consistent with the monensin application phase of the trial (Chapter V).

Weldon (2013) did not observe a difference ($P = 0.66$) in acetate:propionate ratio between Bi and Bt steers. Similarly, no difference was observed (3.86 and 3.76 for Bi and Bt, respectively) for the acetate:propionate ratio in the present study.

Weldon (2013) observed a tendency for molar percentage of butyrate to be greater in Bi than Bt cattle (7.08 vs 6.32 %), however, acetate, propionate, and butyrate were

similar between subspecies throughout the withdrawal period in the present study. The forage fed by Weldon (2013) was a low-quality forage (4.6% CP), while the forage fed in the present study was of better quality (14.3% CP). It is likely that the difference in forage quality had an impact on proportion of VFA, however, further work is warranted to verify the accuracy of this postulation.

Methane-producing activity

Hungate et al. (1960) observed no differences in the proportion of CH₄ in the fermentation gas between subspecies (19.4 and 19.8 μmol·100 μmol of total products⁻¹ for Bi and Bt, respectively). However, during the monensin inclusion phase of this trial (Chapter V) Bi steers had greater *in vitro* CH₄-producing activity than Bt (Chapter V). Following withdrawal, however, observations were similar to Hungate et al. (1960), with no differences observed between the subspecies.

Ruminal ammonia nitrogen

Although Bi steers have been observed to have greater RAN concentration than Bt while consuming LQF (Hunter and Siebert, 1985a; Hennessy et al., 2000), the subspecies difference is diet dependent and is reduced as the dietary-N supply increases (Hunter and Siebert, 1985a). Feeding of adequate N, as in the bermudagrass hay fed during this trial, hinders a subspecies difference and, as expected, concentration of RAN was similar between subspecies. With similar RAN between subspecies, rate of NH₃ production was expectedly similar between subspecies.

Intake

Bos taurus cattle are generally observed to have greater intakes than Bi when consuming forage (Ledger et al., 1970; Frisch and Vercoe, 1977; Hennessy et al., 2000; Habib et al., 2008; Weldon, 2013); however, the difference between subspecies appears to be diet dependent, becoming smaller when lower quality, N-deficient forages are fed (Hunter and Siebert, 1986, 1987). Hunter and Siebert (1986) observed similar intakes when cattle consumed low-quality speargrass forage (3.88% CP); however, when these cattle consumed high-quality lucerne forage (18.44% CP), greater consumption was observed in Bt than Bi (26.3 vs 22.4 g DM/kg BW). Accordingly, because the bermudagrass hay in the present study provided adequate N, FOMI and FNDFI were greater (10.4% and 11.6%, respectively) in Bt than Bi steers.

Digestion

Many studies indicate that Bi cattle may have greater ability to digest low-quality forages than Bt (French, 1940; Phillips et al., 1960; Hunter and Siebert, 1985a; Habib et al., 2008; Weldon, 2013), however these differences tend to be small or non-significant. During the monensin inclusion phase of this trial, no subspecies differences ($P > 0.10$) were observed for OMD or NDFD. Habib et al. (2011) was able to observe a significant ($P \leq 0.05$) difference in steers consuming low-digestibility (58.0%) rice straw, but similar OMD in steers consuming high-digestibility (69.0%) German grass. In the present trial, OMD was expected to be similar between subspecies because of the high (72%) digestibility of the bermudagrass hay and the lack of difference during the monensin inclusion phase. In contrast, however, 14.8% greater OMD and 12.5% greater

NDFD were observed in Bt than Bi steers. Furthermore, when the combined subspecies effects on TOMI and OMD were evaluated as TDOMI, Bt steers were observed to have 27.1% greater TDOMI than Bi steers. Similarly, when TNDFI and NDFD were evaluated as TDNDFI, Bt steers were observed to have 25.0% greater TDNDFI than Bi steers.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Protein supplementation strategies and monensin inclusion were investigated as strategies to potentially improve animal production efficiency while mitigating enteric CH₄ production, thus reducing the economic and environmental impact of cattle production systems. Additionally, *Bos indicus* and *Bos taurus* steers were used to investigate potential differences in response to protein supplementation and monensin inclusion and withdrawal.

When the effect of two sources and levels of protein on ruminal methane-producing activity, rate of NH₃ production, and concentrations of methanogens, protozoa, amino-acid utilizing, and trypticase-metabolizing bacteria were investigated in Bi and Bt steers consuming LQF, subspecies differences were not observed ($P > 0.10$). An effect of protein supplementation was observed ($P \leq 0.01$) in Bi steers at feeding (0 h) and 4 h later for protozoa concentration measured by PCR. An effect of protein supplementation was observed ($P \leq 0.01$) in Bt steers at feeding (0 h) for amino acid-utilizing bacteria and trypticase-metabolizing bacteria and 4 h later for concentration of methanogens and protozoa measured by PCR, and rate of NH₃ production.

When monensin was included in the diet of Bi and Bt steers consuming bermudagrass, subspecies differences were observed ($P \leq 0.10$) for TDOMI, TDNDFI, total VFA, and CH₄-producing activity. An effect of monensin inclusion was observed for FOMI, FNDFI, total VFA, acetate, propionate, acetate:propionate ratio and butyrate.

When monensin was withdrawn from the diet, subspecies differences were observed ($P \leq 0.10$) for FOMI, OMD, TDOMI, FNDFI, NDFD, and TDNDFI. An effect of monensin withdrawal was observed for acetate, propionate, acetate:propionate ratio, butyrate, pH, and RAN.

Results suggest that the difference in ability of Bi and Bt cattle to utilize forage is diet dependent and may be altered by protein supplementation and monensin inclusion to improve production efficiency and mitigate enteric CH₄ production. Effects, however, are diet and subspecies dependent and these factors should be considered when determining the most economical program for a specific beef cattle operation.

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APPENDIX A

CHAPTER II FIGURES AND TABLES

FIGURES

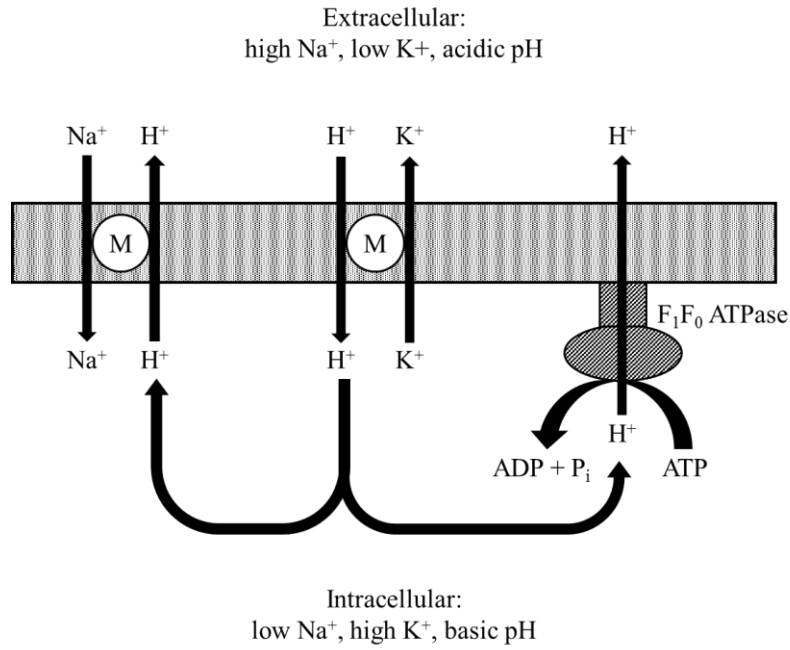


Figure A1. Monensin's (M) mechanism of action.

TABLES

Table A1. Effect of ionophores on *in vivo* ruminant methane production.

Reference	Diet	Animal	Ionophore ¹	Dose	Time ² , d	Inhibition, %	CH ₄ expressed	CH ₄ measurement method
BEEF CATTLE								
Wedegaertner and Johnson, 1983	70% cracked corn and corn silage	Hereford and Herford/Angus steers	M	3 mg/kg MBW/d	21-28	26	% GEI	Respiratory Chamber
Thornton and Owens, 1981	low roughage medium roughage high roughage	steers	M	200 mg/d 200 mg/d 200 mg/d	10-15	15.6 16.4 23.7	kcal/h	Respiratory Chamber
O’Kelly and Spiers, 1992	Lucerne hay ad libitum Lucerne hay (250 g/h)	Brahman steers	M	33 mg/kg feed 33 mg/kg feed	54 51	25.6 0	mL/min	Respiratory Chamber
Rumpler et al., 1986	70% cracked corn, 20% corn silage, 10% supplement	Hereford steers	M	226 mg/d	2-3 12-23	15.0 0	L/d	Respiratory Chamber
			L	226 mg/d	2-3 12-23	11.5 0		
Delfino et al., 1988	90% conc: 10% forage 21 g DM/kg MBW 44 g DM/kg MBW 67 g DM/kg MBW 89 g DM/kg MBW	steers	L	36 mg/kg DM	22	2.8 17.0 0 25.6	% GEI	Respiratory Chamber
Guan et al., 2006	low (12.9%) concentrate high (67.9%) concentrate low (12.9%) concentrate high (67.9%) concentrate	Angus yearling steers	M	33 mg/kg DM	7-28 29-98	30.0 0	L/kg DMI or % GEI	SF ₆
			M	33 mg/kg DM	7-14 15-98	27.0 0		
			M/L: 2 wk rotation	M: 33 mg/kg DM L: 36 mg/kg DM	7-28 29-98	30.0 0		
			M/L: 2 wk rotation	M: 33 mg/kg DM L: 36 mg/kg DM	7-14 15-98	27.0 0		
DAIRY CATTLE								
Odongo et al., 2006	60:40 forage-to-concentrate TMR	lactating Holstein cows	M	24 mg Rumensin premix/kg DM	28-180	6.5	g/d	Hood Calorimetry

Table A1. Continued.

McGinn et al., 2004	75% barley silage, 19% steam flaked barley grain, 6% supplement	Holstein steers	M	33 mg/kg DM	17-21	9.0	% GEI	Respiratory Chamber
Sauer et al., 1998	30% corn silage, 26% alfalfa haylage, 9% hay, 35% TMR concentrate	lactating Holstein cows	M	24 ppm	yr 1: 21.4 yr 2: 5.4	7-28	L/d	Infrared gas analyzer
Mwenya et al., 2005	800 g/kg DM concentrates, 200 g/kg DM mixed hay	Holstein steers	M	30 mg/kg DM	22	16.7	MJ/100 MJ GEI	Respiratory Chamber
Hamilton et al., 2010	37.5% grain mix (steam flaked corn, rolled barley, bakery product), 36.1% alfalfa hay, 26.4% almond hulls, cottonseed, soybean meal, mineral mix	primiparous lactating Holstein cows	M	600 mg/d	14, 60	0	g/hr	Respiratory Chamber
Grainger et al., 2008	Grazing predominantly ryegrass pasture Harvested fresh ryegrass ad libitum and 10 kg/d cracked barley	Holstein-Friesian lactating cows	M	CRC: 12-14.5 mg/kg DMI	25,81,83	0	g/d	SF ₆
					75	0	g/d	Respiratory Chamber
Grainger et al., 2010	Grazing predominantly ryegrass pasture Harvested fresh ryegrass ad libitum and 4 kg/d rolled barley	Holstein-Friesian lactating cows	M	450 mg/d	21, 84	0	g/d	SF ₆
					35, 70	0		Respiratory Chamber
Waghorn et al., 2008	Grazing predominantly ryegrass pasture	Friesian-Holstein lactating cows	M	CRC: 170 mg/d (10.8 mg/kg DMI)	5 40 70	0 4.8 2.0	g/d	SF ₆
SHEEP								
Joyner et al., 1979	50% Alfalfa hay and 50% corn, barley	wethers	M	10 ppm 20 ppm	7-14	25.8 30.7	kcal/d	Respiratory Chamber

¹M = monensin; L = lasalocid²Number of days between beginning ionophore administration and measurement of methane production

APPENDIX B

CHAPTER III FIGURES AND TABLES

TABLES

Table B1. Chemical composition of forage and supplements

Item	Rice straw	L-DIP ¹	H-DIP ²
	----- % DM -----		
OM	84.9	94.5	93.6
CP	4.7	26.7	26.9
NDF	72.8	41.8	35.0
ADF	52.3	19.0	12.4

¹L-DIP = low degradable intake protein supplement (100% Distillers' dried grains)

²H-DIP = high degradable intake protein supplement (69.5% wheat middlings, 30% soybean meal, and 0.5% urea)

Table B2. PCR primers used in this study

Target	Name	Sequence (given in 5' to 3' direction) ¹	Reference
Methanogens	mrcA F	GGTGGTGTGGMGATTCACACAGTAYGC	Denman et al., 2007
	mrcA R	TTCATTGCRTAGTTWGGRTAGTT	
Protozoa	P-SSU-316F	GCTTTCGWTGGTAGTGTATT	Sylvester et al., 2004
	P-SSU-316R	ACTTGCCCTCYAATCGTWCT	

¹W = A or T; R = A or G; Y = C or T

Table B3. Effect of level and source of supplemental protein on rate of ruminal methane production and methanogen concentration in *Bos taurus* and *Bos indicus* steers consuming low-quality forage

Item	Subspecies	Hr	Treatment ¹					SEM ³	Contrast <i>P</i> -value ²					
			CON	L-50	L-100	H-50	H-100		Bi v Bt	h0 v h4	CON v Supp	S × L	50 v 100	H v L
MPN methanogens, log ₁₀ cells/mL	Bi	0	6.71	6.05	6.16	6.55	5.97	0.54	0.80	0.47	0.40	0.94	0.67	0.78
	Bt	0	6.21	6.15	6.61	6.56	6.29	0.37		0.08	0.62	0.84	0.79	0.90
	Bi	4	6.64	6.16	6.45	6.55	7.05	0.54	0.33		0.89	0.37	0.48	0.38
	Bt	4	6.98	6.48	6.85	6.67	7.37	0.37			0.74	0.29	0.15	0.33
PCR Methanogen concentration, log ₁₀ cells/mL	Bi	0	7.17	6.97	6.77	7.01	6.90	0.42	0.64	0.92	0.36	0.70	0.53	0.73
	Bt	0	7.27	7.19	7.24	7.15	7.10	0.38		0.86	0.70	0.66	0.99	0.70
	Bi	4	6.79	6.93	7.17	6.90	6.96	0.42	0.57		0.48	0.57	0.54	0.62
	Bt	4	6.69	7.59	7.35	6.96	7.51	0.38			0.02	0.68	0.52	0.33
Methane production, μmol CH ₄ ·mL ⁻¹ ·h ⁻¹	Bi	0	2.26	2.23	2.34	2.61	2.40	0.32	0.64	0.01	0.72	0.62	0.87	0.50
	Bt	0	2.06	2.30	2.06	2.43	2.51	0.31		0.09	0.46	0.30	0.81	0.35
	Bi	4	1.44	1.67	1.37	1.81	1.95	0.32	0.33		0.48	0.22	0.81	0.29
	Bt	4	1.82	1.90	1.45	1.96	2.09	0.31			0.92	0.18	0.62	0.27

¹Control = 0 mg N/kg BW, L-50 = 50 mg N/kg BW L-DIP, L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP.

²Bi vs Bt = *Bos indicus* versus *Bos taurus*, h0 v h4 = immediately prior to feeding versus 4 h later, CON vs Suppl = control versus supplementation, S × L = effect of increasing N source and increasing N level, 50 vs 100 = 50 mg N/kg BW versus 100 mg N/kg BW, H vs L = effect of both levels of L-DIP compared to both levels of H-DIP.

³SEM = standard error of the mean.

Table B4. Effect of level and source of supplemental protein on protozoa concentration in *Bos taurus* and *Bos indicus* steers consuming low-quality forage

Item	Subspecies	Hr	Treatment ¹					SEM ³	Contrast P-value ²					
			CON	L-50	L-100	H-50	H-100		Bi v Bt	h0 v h4	CON v Suppl	S × L	50 v 100	H v L
PCR Protozoa concentration, log ₁₀ cells/mL	Bi	0	4.10	4.24	4.44	3.74	4.18	0.21	0.37	0.31	0.82	0.11	0.12	0.07
	Bt	0	3.82	3.85	4.01	4.15	4.11	0.22		0.92	0.39	0.46	0.78	0.35
	Bi	4	3.88	4.15	4.75	4.16	4.30	0.21	0.11		0.05	0.17	0.07	0.28
	Bt	4	3.55	4.09	3.99	3.97	4.28	0.22			0.03	0.50	0.63	0.70

¹Control = 0 mg N/kg BW, L-50 = 50 mg N/kg BW L-DIP, L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP.

²Bi vs Bt = *Bos indicus* versus *Bos taurus*, h0 v h4 = immediately prior to feeding versus 4 h later, CON vs Suppl = control versus supplementation, S × L = effect of increasing N source and increasing N level, 50 vs 100 = 50 mg N/kg BW versus 100 mg N/kg BW, H vs L = effect of both levels of L-DIP compared to both levels of H-DIP.

³SEM = standard error of the mean.

FIGURES

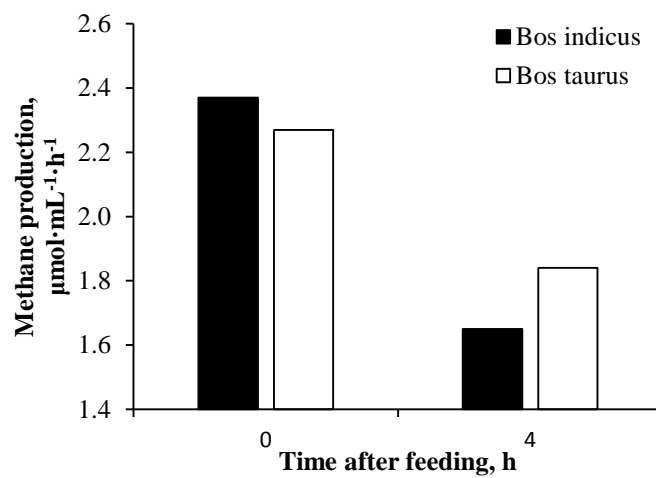


Figure B1. Effect of protein supplement amount and degradability on methane production in *Bos indicus* (Bi) and *Bos taurus* (Bt) steers. Effect of time after feeding in Bi ($P = 0.01$) and a tendency for effect of time after feeding in Bt steers ($P = 0.09$).

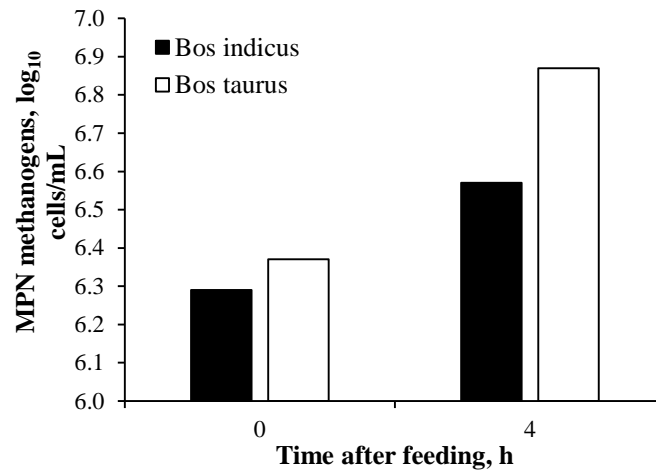


Figure B2. Effect of protein supplement amount and degradability on MPN methanogens in *Bos indicus* (Bi) and *Bos taurus* (Bt) steers. Tendency for effect of time after feeding in Bt steers ($P = 0.08$).

APPENDIX C

CHAPTER IV FIGURES AND TABLES

TABLES

Table C1. Chemical composition of forage and supplements

Item	Rice straw	L-DIP ¹	H-DIP ²
	----- % DM -----		
OM	84.9	94.5	93.6
CP	4.7	26.7	26.9
NDF	72.8	41.8	35.0
ADF	52.3	19.0	12.4

¹L-DIP = low degradable intake protein supplement (100% Distillers' dried grains)

²H-DIP = high degradable intake protein supplement (69.5% wheat middlings, 30% soybean meal, and 0.5% urea)

Table C2. Effect of level and source of supplemental protein on rate of ruminal ammonia production and concentrations of amino acid-utilizing and trypticase-metabolizing bacteria in *Bos taurus* and *Bos indicus* steers consuming low-quality forage

Item	Subspecies	Hr	Treatment ¹					SEM ³	Contrast <i>P</i> -value ²					
			CON	L-50	L-100	H-50	H-100		Bi v Bt	h0 v h4	CON v Supp	S × L	50 v 100	H v L
Ammonia, μmol·mL ⁻¹ ·h ⁻¹	Bi	0	32.79	44.62	34.64	30.97	45.59	9.70	0.91	0.63	0.53	0.77	0.79	0.88
	Bt	0	30.06	29.74	37.89	29.53	48.29	8.34		0.41	0.50	0.43	0.11	0.54
	Bi	4	33.45	40.49	40.54	42.88	50.15	8.73	0.78		0.29	0.42	0.66	0.48
	Bt	4	31.91	33.72	33.46	42.32	58.37	7.28			0.24	0.02	0.30	0.04
Amino acid-utilizing bacteria, log ₁₀ cells/mL	Bi	0	6.14	5.89	6.21	6.05	6.01	0.36	0.54	0.85	0.67	0.96	0.42	0.72
	Bt	0	5.25	5.74	6.00	5.96	6.41	0.23		0.77	<0.01	0.11	0.09	0.13
	Bi	4	6.08	6.19	6.22	5.88	5.88	0.36	0.28		0.92	0.40	0.97	0.38
	Bt	4	6.04	6.08	5.81	5.37	5.85	0.23			0.23	0.32	0.58	0.11
Trypticase- metabolizing bacteria, log ₁₀ cfu/mL	Bi	0	7.59	7.95	7.56	7.06	7.47	0.23	0.91	<0.01	0.76	0.14	0.96	0.04
	Bt	0	8.02	7.52	7.42	7.27	7.32	0.22		<0.01	0.02	0.56	0.91	0.46
	Bi	4	7.01	7.27	7.26	7.03	7.01	0.23	0.41		0.60	0.30	0.93	0.28
	Bt	4	6.97	6.84	6.96	7.00	7.20	0.22			0.91	0.40	0.51	0.40

¹Control = 0 mg N/kg BW, L-50 = 50 mg N/kg BW L-DIP, L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP.

²Bi vs Bt = *Bos indicus* versus *Bos taurus*, h0 v h4 = immediately prior to feeding versus 4 h later, CON vs Supp = control versus supplementation, S × L = effect of increasing N source and increasing N level, 50 vs 100 = 50 mg N/kg BW versus 100 mg N/kg BW, H vs L = effect of both levels of L-DIP compared to both levels of H-DIP.

³SEM = standard error of the mean.

FIGURES

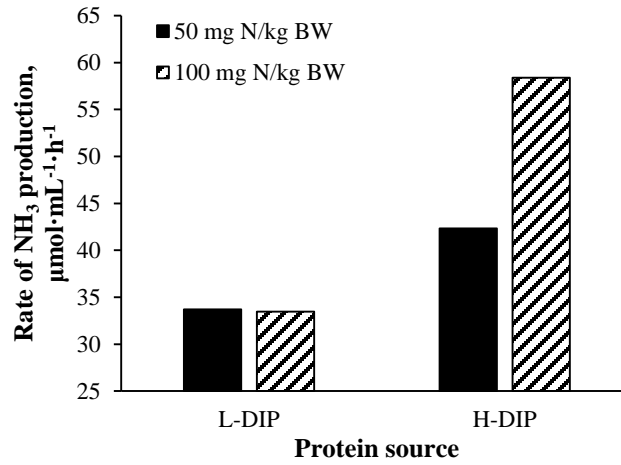


Figure C1. Effect of protein supplement amount and degradability on rate of *in vitro* ammonia production in *Bos taurus* (Bt) steers at h 4. L-50 = 50 mg N/kg BW L-DIP; L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP. Source × level interaction ($P = 0.02$).

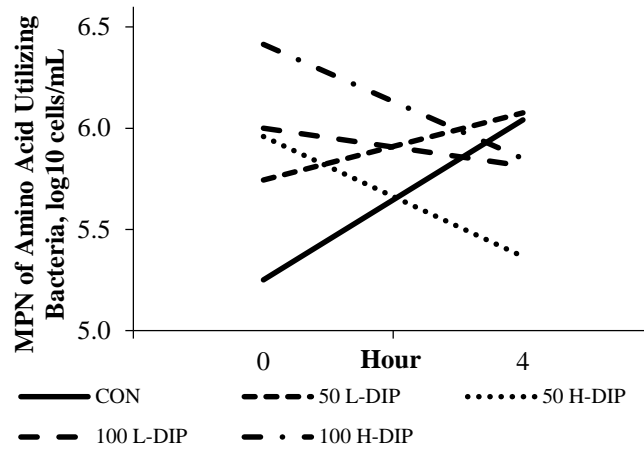


Figure C2. Effect of protein supplement amount and degradability on most probable number of amino acid utilizing bacteria in *Bos taurus* (Bt) steers. CON = 0 mg N/kg BW; L-50 = 50 mg N/kg BW L-DIP; L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP. Treatment \times time interaction ($P = 0.04$).

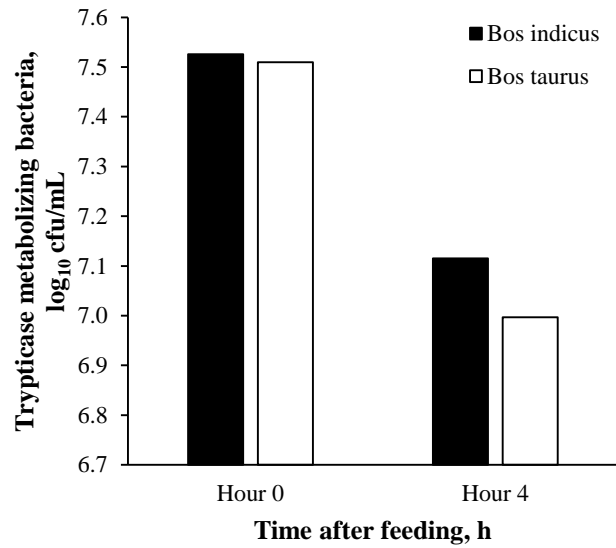


Figure C3. Effect of protein supplement amount and degradability on concentration of trypticase-metabolizing bacteria in *Bos indicus* (Bi) and *Bos taurus* (Bt) steers. CON = 0 mg N/kg BW; L-50 = 50 mg N/kg BW L-DIP; L-100 = 100 mg N/kg BW L-DIP, H-50 = 50 mg N/kg BW H-DIP, H-100 = 100 mg N/kg BW H-DIP. Effect of time after feeding Bi ($P < 0.01$) and Bt ($P < 0.01$).

APPENDIX D

CHAPTER V FIGURES AND TABLES

TABLES

Table D1. Chemical composition of forage and supplement

Item	Bermudagrass hay	Distillers' dried grains with solubles
	----- % DM -----	
OM	91.4	90.9
CP	13.7	31.2
NDF	77.3	43.8
ADF	38.8	17.5
Acid detergent insoluble ash	2.34	0.40

Table D2. Effect of monensin inclusion on intake of steers consuming bermudagrass hay

	Subspecies	Treatment ¹		SEM ³	P-values ²	
		CON	MON		Bi vs Bt	CON vs MON
OMI, g/kg BW						
Forage	Bi	18.6	19.8	0.69	0.16	0.08
	Bt	20.3	20.9			
DDG	Bi	1.8	1.8	-	-	-
	Bt	1.8	1.8			
Total	Bi	20.5	21.6	0.68	0.16	0.07
	Bt	22.1	22.7			
Digestible	Bi	11.5	12.9	0.90	0.04	0.71
	Bt	14.7	14.0			
NDFI, g/kg BW						
Forage	Bi	15.8	16.8	0.59	0.16	0.08
	Bt	17.3	17.7			
DDG	Bi	0.9	0.9	-	-	-
	Bt	0.9	0.9			
Total	Bi	16.7	17.7	0.58	0.16	0.08
	Bt	18.1	18.6			
Digestible	Bi	10.4	11.4	0.63	0.02	0.73
	Bt	13.0	12.4			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.21$.

³SEM = standard error of the mean.

Table D3. Effect of monensin inclusion on digestion in steers consuming bermudagrass hay.

	Subspecies	Treatment ¹			P-values ²	
		CON	MON	SEM ³	Bi vs Bt	CON vs MON
Total tract digestion, %						
OM	Bi	56.1	59.8	4.52	0.28	0.97
	Bt	65.0	61.6			
NDF	Bi	62.4	64.9	3.68	0.22	0.89
	Bt	70.4	67.0			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.36$.

³SEM = standard error of the mean.

Table D4. Effect of monensin inclusion on volatile fatty acids in steers consuming bermudagrass hay.

	Subspecies	Treatment ¹		SEM ³	P-values ²	
		CON	MON		Bi vs Bt	CON vs MON
Total VFA, mM	Bi	64.16	61.33	2.24	0.28	0.01
	Bt	68.82	62.68			
Acetate:Propionate	Bi	4.44	3.97	0.15	0.22	<0.01
	Bt	4.25	3.72			
Molar percentage, % Acetate	Bi	72.80	71.72	0.53	0.18	0.02
	Bt	72.22	70.78			
Propionate	Bi	16.57	18.20	0.57	0.24	<0.01
	Bt	17.23	19.13			
Butyrate ⁴	Bi	7.86	7.33	0.32	0.52	0.04
	Bt	7.66	7.05			
Isobutyrate	Bi	0.90	0.90	0.07	0.76	0.49
	Bt	0.88	0.97			
Valerate	Bi	0.85	0.81	0.03	0.26	0.14
	Bt	0.89	0.85			
Isovalerate	Bi	1.02	1.05	0.08	0.12	0.39
	Bt	1.13	1.22			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.28$; monensin × hour after feeding interactions $P \geq 0.29$; subspecies × hour after feeding interactions $P \geq 0.54$.

³SEM = standard error of the mean.

⁴Subspecies × monensin × hour after feeding interaction ($P = 0.09$).

Table D5. Effect of monensin inclusion on ruminal fermentation parameters in steers consuming bermudagrass hay.

	Subspecies	Treatment ¹		SEM ³	P-values ²	
		CON	MON		Bi vs Bt	CON vs MON
pH	Bi	6.68	6.66	0.07	0.55	0.35
	Bt	6.56	6.69			
Ruminal ammonia, mM ⁴	Bi	1.73	1.89	0.11	0.50	0.32
	Bt	1.70	1.75			
Ammonia, $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$	Bi	0.06	0.05	0.02	0.11	0.33
	Bt	0.06	0.12			
Methane production, $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$	Bi	22.48	20.25	2.24	0.07	0.19
	Bt	18.76	14.49			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.14$; subspecies × monensin × hour after feeding interactions $P \geq 0.27$; monensin × hour after feeding interactions $P = 0.39$; subspecies × hour after feeding interactions $P \geq 0.39$.

³SEM = standard error of the mean.

⁴subspecies × hour after feeding interaction ($P = 0.04$).

FIGURES

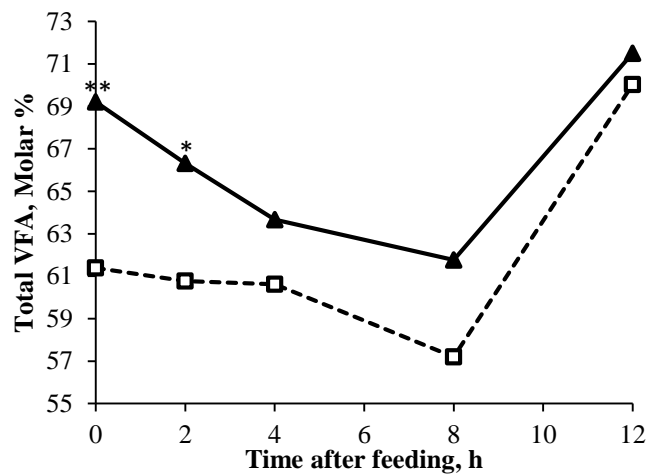


Figure D1. Effect of monensin total VFA concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (◻) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$; * = $P \leq 0.10$. Effect of hour after feeding ($P < 0.01$).

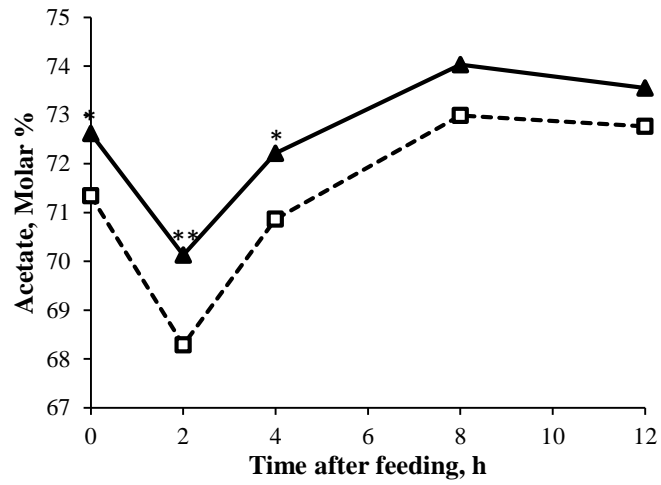


Figure D2. Effect of monensin on molar percentage of acetate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$; * = $P \leq 0.10$. Effect of hour after feeding ($P < 0.01$).

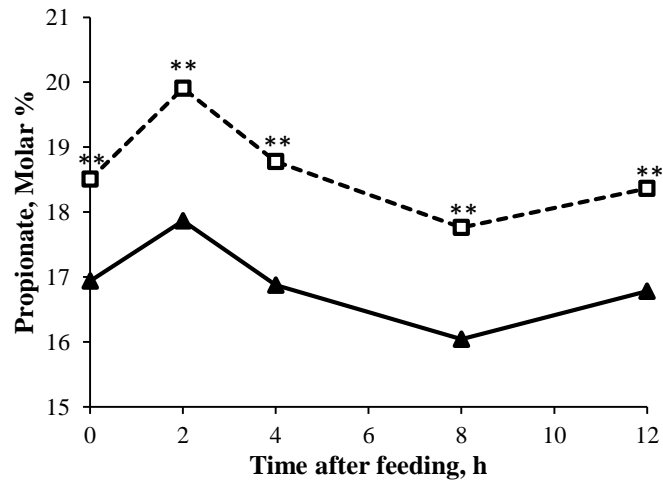


Figure D3. Effect of monensin on molar percentage of propionate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Effect of hour after feeding ($P < 0.01$).

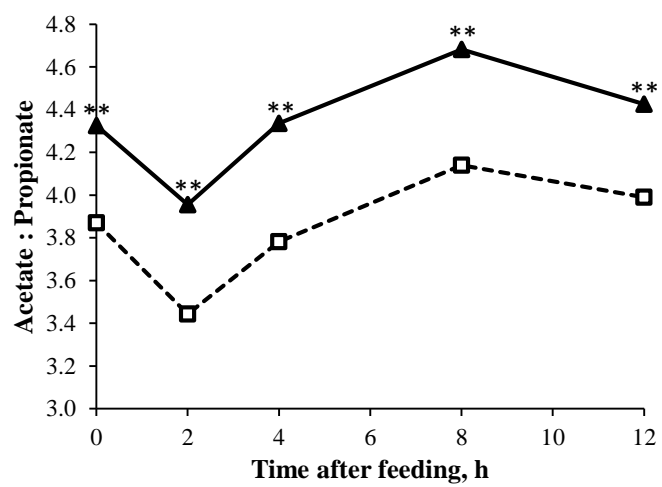


Figure D4. Effect of monensin on the acetate:propionate ratio in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Effect of hour after feeding ($P < 0.01$).

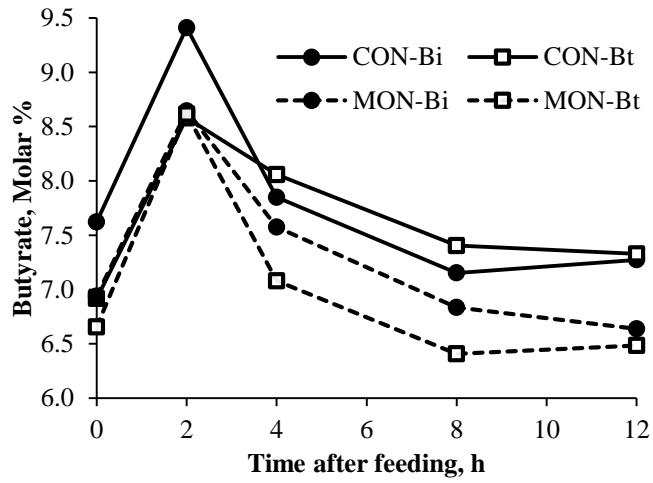


Figure D5. Effect of monensin and subspecies on butyrate in the ruminal fluid of steers consuming bermudagrass hay. CON (\blacktriangle) = $0 \text{ mg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$ monensin, MON (\square) = $200 \text{ mg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), Bi = *Bos indicus*, Bt = *Bos taurus*. Subspecies \times monensin \times hour after feeding interaction ($P = 0.09$).

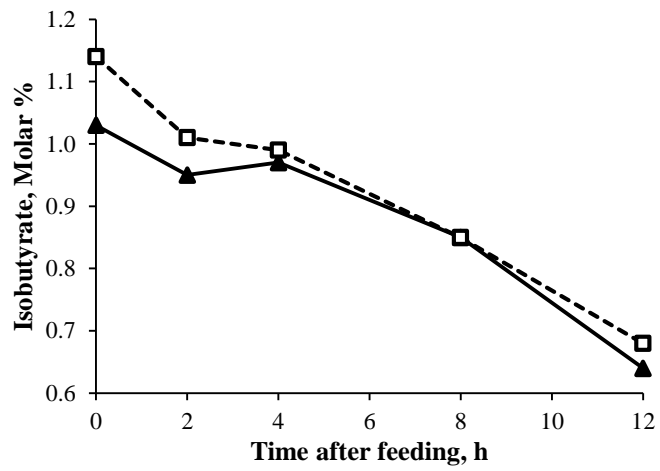


Figure D6. Effect of monensin on isobutyrate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Effect of hour after feeding ($P < 0.01$).

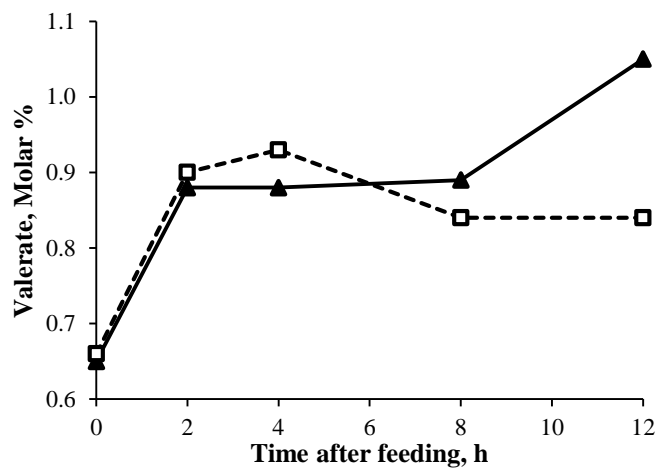


Figure D7. Effect of monensin on valerate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Effect of hour after feeding ($P < 0.01$).

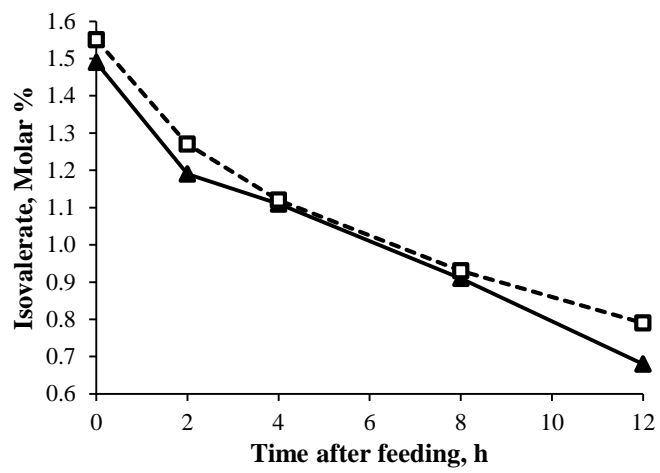


Figure D8. Effect of monensin on isovalerate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Effect of hour after feeding ($P < 0.01$).

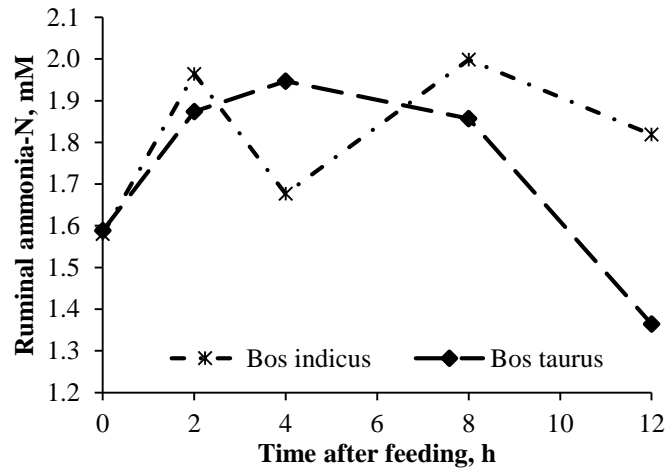


Figure D9. Effect of monensin on ruminal ammonia-N in the ruminal fluid of steers consuming bermudagrass hay. CON = 0 mg·hd⁻¹·d⁻¹ monensin, MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Subspecies × hour after feeding interaction ($P = 0.04$).

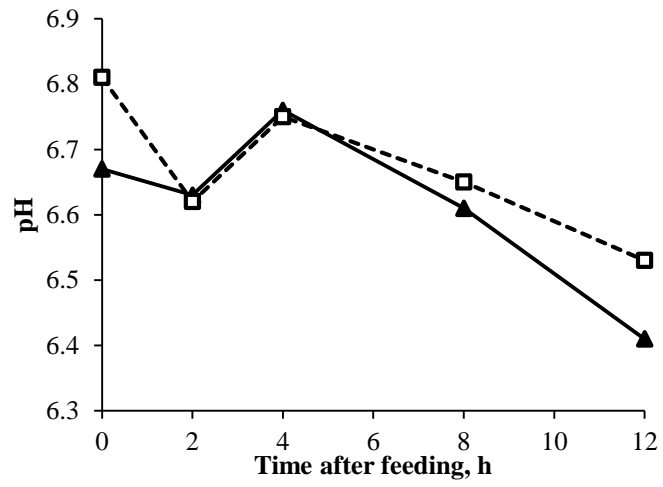


Figure D10. Effect of monensin on pH in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Effect of hour after feeding ($P < 0.01$).

APPENDIX E

CHAPTER VI FIGURES AND TABLES

TABLES

Table E1. Chemical composition of forage and supplement

Item	Bermudagrass hay	Distillers' dried grains with solubles
	----- % DM -----	
OM	92.1	91.1
CP	14.3	33.3
NDF	72.3	42.6
ADF	36.9	19.9
Acid detergent insoluble ash	1.63	0.31

Table E2. Effect of monensin withdrawal on volatile fatty acids in steers consuming bermudagrass hay

	Subspecies	Treatment ¹			P-values ²			
		CON	MON	SEM ³	Bi vs Bt	CON vs MON	Day	Trt×Day
Total VFA, mM	Bi	72.52	69.01	1.69	0.36	0.43	<0.01	0.18
	Bt	71.93	72.68					
Acetate:Propionate	Bi	3.91	3.82	0.07	0.23	0.03	<0.01	0.01
	Bt	3.84	3.69					
Molar percentage, %								
Acetate	Bi	69.58	69.60	0.32	0.61	0.30	0.30	0.01
	Bt	69.65	69.13					
Propionate	Bi	17.83	18.32	0.27	0.20	<0.01	<0.01	<0.01
	Bt	18.21	18.83					
Butyrate	Bi	9.76	9.17	0.22	0.30	<0.01	<0.01	<0.01
	Bt	9.37	8.97					
Isobutyrate	Bi	0.81	0.83	0.03	0.21	0.05	<0.01	0.10
	Bt	0.74	0.83					
Valerate	Bi	1.05	1.10	0.05	0.99	0.06	0.06	0.19
	Bt	1.03	1.12					
Isovalerate	Bi	0.96	0.98	0.03	0.02	0.04	0.04	0.27
	Bt	0.99	1.12					

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; Trt×Day = monensin × day after withdrawal interaction; subspecies × monensin interaction $P \geq 0.11$; subspecies × day after withdrawal interaction $P \geq 0.29$; subspecies × monensin × day after withdrawal interaction $P \geq 0.26$.

³SEM = standard error of the mean.

Table E3. Effect of monensin withdrawal on ruminal fermentation parameters in steers consuming bermudagrass hay

	Subspecies	Treatment ¹			P-values ²			
		CON	MON	SEM ³	Bi vs Bt	CON vs MON	Day	Trt×Day
pH	Bi	6.51	6.55	0.03	0.08	0.04	<0.01	0.03
	Bt	6.42	6.50					
Ruminal ammonia, mM	Bi	2.17	1.82	0.07	0.33	<0.01	<0.01	0.52
	Bt	2.00	1.85					
Ammonia, $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$	Bi	0.28	0.28	0.01	0.92	0.69	<0.01	0.23
	Bt	0.28	0.28					
Methane production, $\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$	Bi	20.21	19.82	0.71	0.23	0.81	<0.01	0.86
	Bt	20.95	21.06					

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; Trt×Day = monensin × day after withdrawal interaction; subspecies × monensin interaction $P \geq 0.16$; subspecies × day after withdrawal interaction $P \geq 0.47$; subspecies × monensin × day after withdrawal interaction $P \geq 0.18$.

³SEM = standard error of the mean.

Table E4. Effect of monensin withdrawal on intake in steers consuming bermudagrass hay

	Subspecies	Treatment ¹		SEM ³	P-values ²	
		CON	MON		Bi vs Bt	CON vs MON
OMI, g/kg BW						
Forage	Bi	19.14	19.29	0.84	0.09	0.98
	Bt	21.30	21.12			
DDG	Bi	1.82	1.82	-	-	-
	Bt	1.80	1.80			
Total	Bi	20.97	21.11	0.85	0.10	0.98
	Bt	23.09	22.91			
Digestible	Bi	12.75	13.72	0.50	<0.01	0.70
	Bt	17.10	16.53			
NDFI, g/kg BW						
Forage	Bi	14.90	14.98	0.59	0.04	0.66
	Bt	16.93	16.43			
DDG	Bi	0.85	0.85	-	-	-
	Bt	0.84	0.84			
Total	Bi	15.76	15.84	0.60	0.05	0.66
	Bt	17.77	17.27			
Digestible	Bi	10.05	10.62	0.37	<0.01	0.74
	Bt	13.09	12.75			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.29$.

³SEM = standard error of the mean.

Table E5. Effect of monensin withdrawal on digestion in steers consuming bermudagrass hay

	Subspecies	Treatment ¹		SEM ³	P-values ²	
		CON	MON		Bi vs Bt	CON vs MON
Total tract digestion, %						
OM	Bi	61.11	64.96	2.27	<0.01	0.45
	Bt	72.45	72.30			
NDF	Bi	63.92	67.28	2.04	<0.01	0.42
	Bt	73.76	73.91			

¹CON = 0 mg·hd⁻¹·d⁻¹ monensin; MON = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN).

²Bi vs Bt = *Bos indicus* versus *Bos taurus*; CON vs MON = control versus monensin; subspecies × monensin interactions $P \geq 0.94$.

³SEM = standard error of the mean.

FIGURES

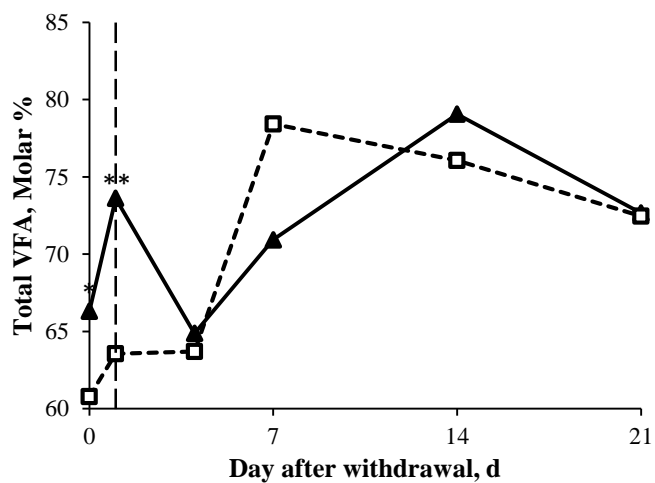


Figure E1. Effect of monensin withdrawal on total VFA concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$, * = $P \leq 0.10$. Effect of day after monensin withdrawal ($P < 0.01$).

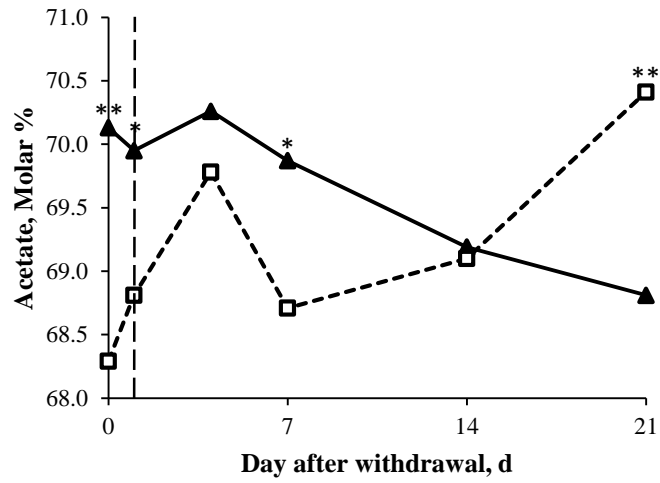


Figure E2. Effect of monensin withdrawal on acetate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$, * = $P \leq 0.10$. Monensin × day after monensin withdrawal interaction ($P = 0.01$).

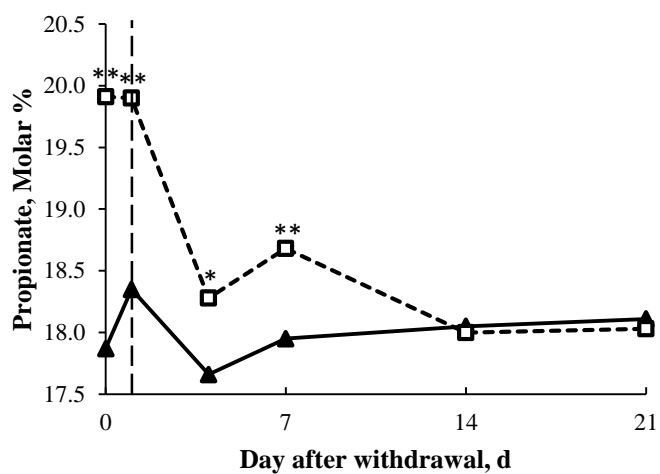


Figure E3. Effect of monensin withdrawal on propionate in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$; * = $P \leq 0.10$. Monensin × day after monensin withdrawal interaction ($P < 0.01$).

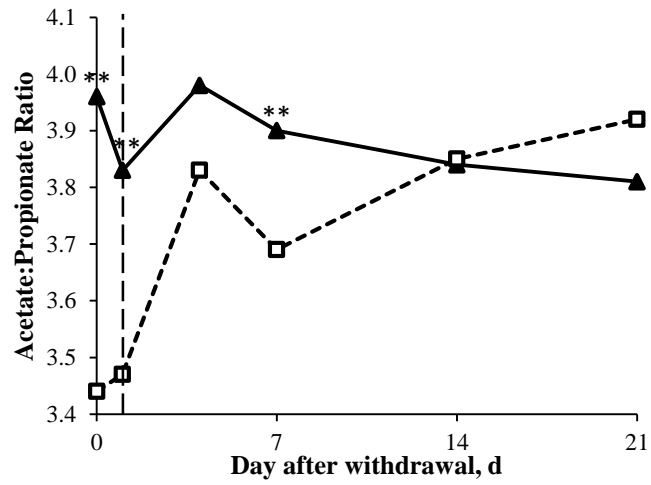


Figure E4. Effect of monensin withdrawal on the acetate:propionate ratio in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Monensin × day after monensin withdrawal interaction ($P = 0.01$).

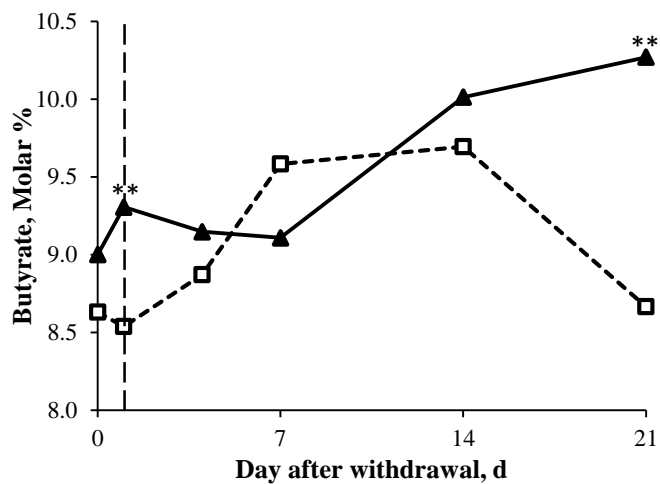


Figure E5. Effect of monensin withdrawal on butyrate concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Monensin × day after monensin withdrawal interaction ($P < 0.01$).

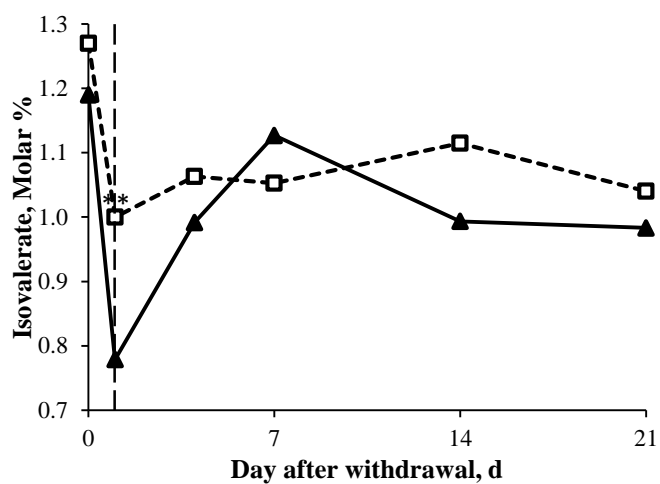


Figure E6. Effect of monensin withdrawal on isovalerate concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (◻) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Effect of day after monensin withdrawal ($P = 0.04$).

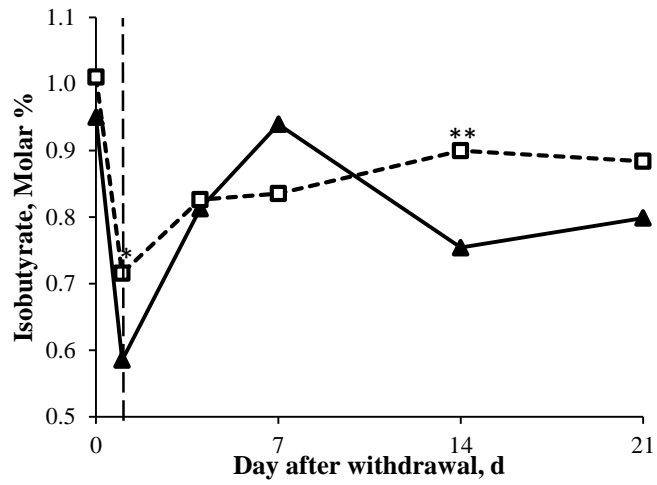


Figure E7. Effect of monensin withdrawal on isobutyrate concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (\blacktriangle) = $0 \text{ mg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$ monensin, MON (\square) = $200 \text{ mg} \cdot \text{hd}^{-1} \cdot \text{d}^{-1}$ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$, * = $P \leq 0.10$. Monensin \times day after monensin withdrawal ($P = 0.10$).

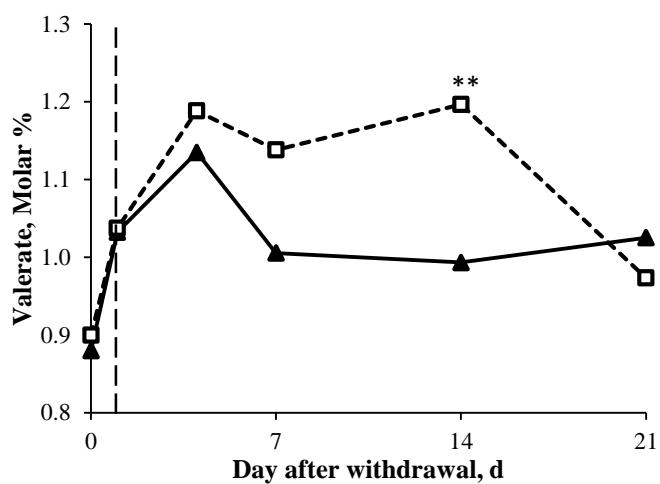


Figure E8. Effect of monensin withdrawal on valerate concentration in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN) ** = $P \leq 0.05$. Effect of day after monensin withdrawal ($P = 0.06$).

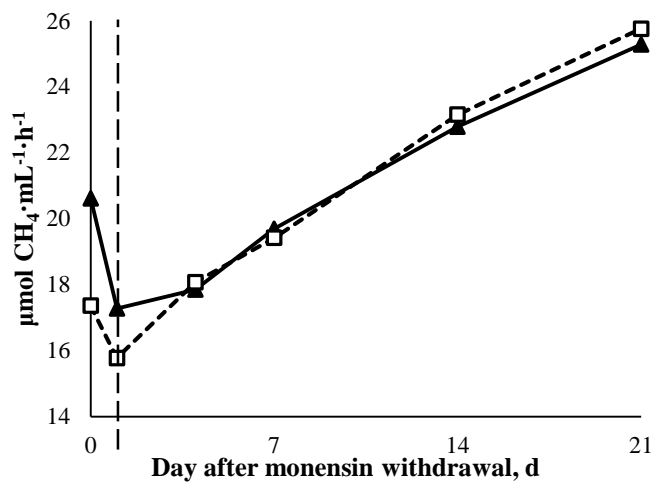


Figure E9. Effect of monensin withdrawal on methane-producing activity in the ruminal fluid steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN). Effect of day after monensin withdrawal ($P < 0.01$).

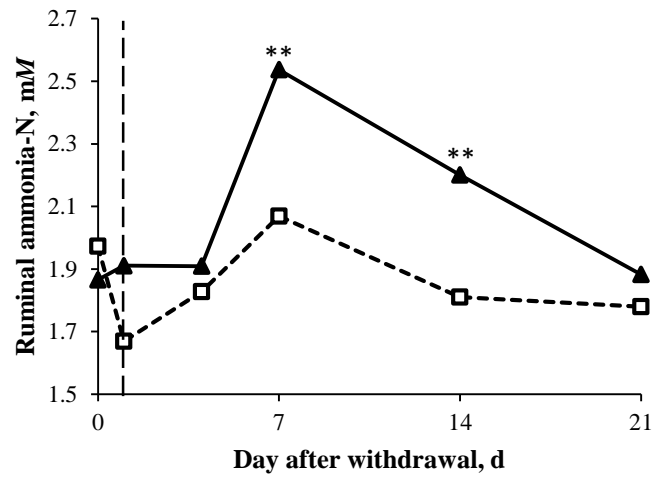


Figure E10. Effect of monensin withdrawal on ruminal ammonia-N in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$). Effect of day after monensin withdrawal ($P < 0.01$).

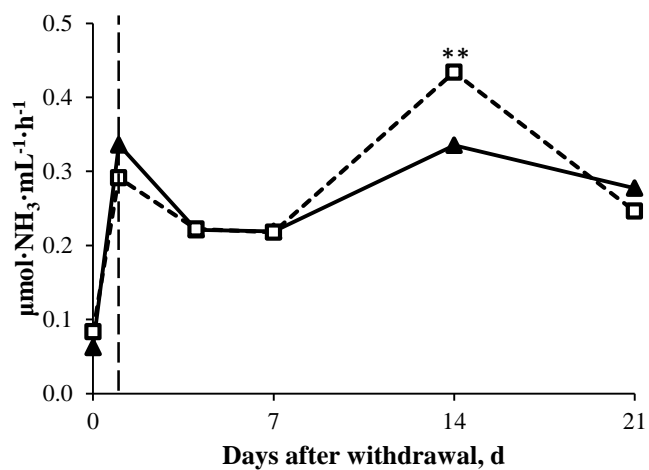


Figure E11. Effect of monensin withdrawal on rate of ruminal ammonia production in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (◻) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Effect of day after monensin withdrawal ($P < 0.01$).

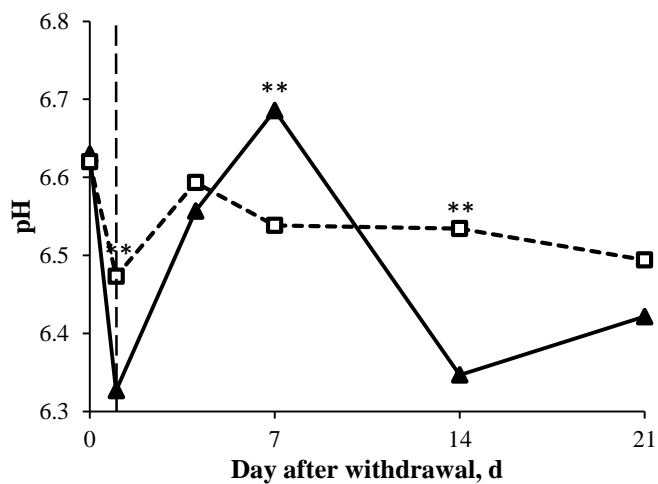


Figure E12. Effect of monensin withdrawal (0 or 200 mg·hd⁻¹·d⁻¹; Rumensin® 90; Elanco Animal Health, Greenfield, IN) on pH in the ruminal fluid of steers consuming bermudagrass hay. CON (▲) = 0 mg·hd⁻¹·d⁻¹ monensin, MON (□) = 200 mg·hd⁻¹·d⁻¹ monensin (Rumensin® 90; Elanco Animal Health, Greenfield, IN), ** = $P \leq 0.05$. Monensin \times day after monensin withdrawal interaction ($P = 0.03$).