UTILIZATION OF NATURAL FEED ADDITIVES TO OPTIMIZE RUMEN EFFICIENCY IN HIGH-ROUGHAGE DIETS

A Thesis

by

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ABSTRACT

In the last two decades, two major public perceptions have been assigned to animal agriculture: antibiotics and ‘non-natural’ products in domestic animal diets are hazardous to human health, and increased greenhouse gas concentrations are the result of cattle production. Therefore, the exploration of natural feed additives and their effects on ruminant performance and resultant greenhouse gas emissions could identify possible alternatives to replace these products. In the first trial, I investigated the utilization of seven encapsulation methods of branched-chain volatile fatty acids (BCVFA) on in vitro gas production dynamics of *Brachiaria brizantha* hay incubated with or without the addition of casein and cysteine (C&C) as a nitrogen source. The inclusion of C&C altered fermentation parameters of salts of BCVFA, indicating that the optimal level of inclusion is dependent on N availability. This trial indicated that the utilization of S1 salt favored maximal total digestible nutrients (TDN) compared to other combinations of encapsulation materials. The utilization of natural plant secondary compounds as feed additives in animal nutrition has been extensively studied because of their ability to modify digestive and metabolic functions. Condensed tannin (CT) supplementation can potentially alter ruminal fermentation and mitigate methane (CH_4) emissions. In the second trial, I evaluated the effect of quebracho CT (QT) extract at four levels (0, 1, 2 and 3% of dry matter; QT0, QT1, QT2, and QT3) of inclusion within a roughage-based diet on overall fermentability and CH_4 production utilizing the in vitro gas production technique. In situ dry matter digestibility increased linearly ($P = 0.048$) as QT inclusion increased. Total volatile fatty acid concentration did not differ among the treatments ($P = 0.470$); however, ruminal NH_3 and blood urea nitrogen (BUN) decreased linearly as QT inclusion increased ($P < 0.075$), suggesting a reduction in

protein deamination in the rumen. CH_4 production was reduced ($P = 0.001$) for the highest level of inclusion QT3 compared to no QT and QT2 on Days 0 and 28, respectively. Moreover, CH_4 production per gram of neutral detergent fiber (NDF) digested tended ($P = 0.054$) to decrease linearly as QT inclusion increased. Thus, the inclusion of QT appears to improve animal performance and reduce CH_4 emissions. However, future studies are required to determine the practicability of QT utilization.

DEDICATION

I would like to dedicate this thesis to my family, which has always encouraged me to pursue my dreams. I love you all.

Rosimeire and Ailton, missing you have been so hard, but the support and belief on myself have gotten here. Thank you. Love you forever.

“Rosimeire e Ailton, a saudade nunca foi tão difícel, mas o apoio e todo o suporte me fizeram chegar aqui, obrigado por tudo. Amo vocês para sempre.”

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1.INTRODUCTION AND LITERATURE REVIEW

1.1. Future Challenges for Ruminant Production

Ruminant livestock systems have a unique characteristic that include producing human-edible products (HEP) from human-inedible feedstuffs such as grasses and agro-industrial byproducts. The main tool that provides ruminants this skill is the reticulum-rumen anaerobic fermentation chamber, where microbes can convert the feed into energy and protein source for the host by producing volatile fatty acids (VFA) and microbial crude protein (MCP). However, the fermentation process in the rumen by the microbes generates waste products such as methane (CH_4), carbon dioxide (CO_2), hydrogen sulfide (H_2S), and ammonia (NH_3). Although, the production of these gaseous end-products preserves ruminal ecosystems equilibrium, the process to produce as well as to release them is responsible for energy losses and contribute to the greenhouse gas (GHG) emission. Holter and Young (1992) estimated that enteric methane production could represent a loss of about 6% of the gross energy (GE) consumed by the animal.

The livestock sector has been blamed for significant contributions to GHG emissions globally. Smith et al. (2014) projected that domestic ruminants accounted for about 56% of the total non-CO_2 GHG emissions in 2005, with the enteric fermentation in domestic livestock animals responsible for 40% of this emission (EPA 2011; Tubiello et al. 2014). Projections of population growth indicate that food production needs to be increased by 50% to meet the demand of global nutrients in 2050 (FAO 2017). The demand to increase food production and decrease methane emission and nitrogen excretion from the ruminant livestock sector has led to intense research of feed additives to improve rumen fermentation and decrease methane production and the energy loss caused by it (Cobellis et al. 2016). However, the vast majority of

studies focused on confined settings using grain-based diets (Carvalho et al. 2017). Unfortunately, this is not representative of the impending issues as approximately 70% of the world`s cattle population resides in tropical and subtropical regions (Cooke et al. 2020) that rely on the conversion of forage fiber into human products (Tedeschi et al. 2012).

1.2. Rumen Efficiency

1.2.1. Rumen Ecosystem

The ruminant stomach is composed of four compartments: the rumen, reticulum, omasum, and abomasum, which comprises more than 50% of the digestive tract in the ruminants. The rumen is a complex ecosystem that behaves like an anaerobic fermentation chamber that makes ruminants different from all other animal species. The ecosystem of the rumen is composed of many different classes of microorganisms, Russell (2002) hypothesized that 1 mm of ruminal fluid contains about 10^{10} bacteria, 10^{6} fungi, and 10^{7} protozoa. The vast number of microbial species in the rumen is the key to the degradation of feedstuff entering the rumen. In summary, microbes supply the host animal with VFA and MCP, as energy and protein source, from the digestion of carbohydrates and protein existing in the feed. The efficiency of which the microbes provide these fermentation end products is mostly dependent upon the diet consumed by the animal.

Rumen bacteria can be generally divided into two major groups, those that ferment fiber carbohydrates (FC), and the other group that ferments nonfiber carbohydrates (NFC) (Russell et al. 1992). Paloheimo and Paloheimo (1949) registered several substances in the NFC group, such as starch, sugars, inulins, glucosides, dextrins, pectins, and others, these substrates have a rapid degradation rate in the rumen compared to the FC group (hemicellulose, cellulose, and lignin) (Tedeschi and Fox 2020). Other researchers have proposed a third group of bacteria, known as

obligate amino acid (OAA) fermenters, or hyper-ammonia producing bacteria (Krause and Russell 1996). However, their impact on ruminal fermentation and the model of growth of these microorganisms have not been well studied (Rychlik and Russell 2000). The microbiome present in the rumen is continuously changing. The presence of these microorganisms are controlled by the availability of substrate (e.g., carbohydrates, protein, lipids), how often that substrate get into the rumen and the microbes can utilize that for growth, as well as the ruminal environment, each class of microorganisms has a specific condition that is preferable for their growth (e.g., pH, and osmolality) (Tedeschi and Fox 2020).

The FC fermenting bacteria have ammonia as the primary N source, since it has partial the capacity to utilize amino-N for growth, and cannot synthesize ammonia from amino N sources, the depletion of ammonia in the rumen impacts the growth of this group of bacteria and therefore limit ruminal fiber fermentation (Allison 1969; Tedeschi and Fox 2020). Additionally, cellulolytic bacteria also require the presence of BCVFA to growth (Bryant and Doetsch 1955; Allison and Bryant 1963; Dehority et al. 1967), the presence of BCVFA in the rumen is produced from degradation products of branched-chain amino acids (BCAA: valine, leucine, and isoleucine, respectively) (Andries et al. 1987). On the other hand, NFC-degrading bacteria utilize amino-N as the primary N source, and can also utilize ammonia or produce amino-N from it. In contrast, the reduction of ammonia-N in the rumen does not compromise NFC bacteria growth, and NFC fermentation severely (Van Kessel and Russell 1996). Therefore, the rate of microbial growth is directly dependent on the availability of N sources in the rumen; thus, when N is limited, fiber carbohydrate degradation in the rumen is depressed to the level of N allows microbial growth (Tedeschi et al. 2000b).

Rumen protozoa are comprised of two groups: flagellates and ciliated. Protozoa have substantial size, high motility, and are predominantly anaerobic. Due to their large size, ruminal protozoa can contribute up to 50% of the biomass in the rumen. The ciliated group is the main contributor to overall protozoa digestion of soluble sugars, structural carbohydrates, and starch granules, whereas flagellates have none or insignificant effects on rumen processes. Additionally, ciliated protozoa commonly present a symbiotic relationship with methanogens in the rumen, and its population has been positively associated with CH_4 production (Van Zijderveld et al. 2010). Although protozoa compromise a large portion of the rumen microflora biomass, their role in ruminal fermentation is not pivotal as the bacteria, and its existence seems not to be essential to the animal host to survive (Williams and Coleman 1997). Nevertheless, the removal of protozoa from the rumen negatively impacts fiber and protein digestibly, VFA production in the rumen, and microbial flow to the duodenum (Newbold et al. 2015).

1.2.2. Branched-chain Volatile Fatty Acids

Branched-chain volatile fatty acids (isobutyric, isovaleric, and 2-methylbutyric) are primarily derived from dietary sources or deamination of branched-chain amino acids. The BCVFA are required for several ruminal bacteria, especially the fiber-degrading microorganisms (Cummins and Papas 1985). Hence, a minimal level of BCVFA in the rumen is essential for digestion of structural carbohydrates, and thus, synthesis of microbial protein in fiber diets (Wang et al. 2012). The addition of BCVFA in the rumen in vitro studies increases microbial protein synthesis (Hume 1970; Cummins and Papas 1985) plant cell wall fermentation (Gorosito et al. 1985) and dry matter digestion (Mir et al. 1986). Other in vivo studies indicated that the supplementation of BCVFA improved nitrogen retention, feed intake, VFA concentration, and overall digestibility of dry matter (DM), organic matter (OM), and fiber portion (Hungate and

Dyer 1956; Misra and Thakur 2001; Wang et al. 2012). Liu et al. (2014) supplemented three levels of isovalerate (8.4, 16.8, and 25.2g/d) to growing steers consuming a corn-stover-based diet and reported a concurrent increase in the cellulolytic bacteria population while CH_4 production was reduced with the addition of isovalerate. Because microbial protein can represent the majority of the metabolizable protein (MP) utilized by the animal (Tedeschi and Fox 2020), due to its high digestibility in the small intestine, provision of BCVFA can not only impact fiber fermentation but also enhance microbial growth, and therefore, increase MP supply for the animal (Tedeschi et al. 2000a, 2015; Fox and Tedeschi 2003).

1.2.3. Condensed Tannins

1.2.3.1 Classification of Tannins

Plant secondary metabolites are chemicals structures developed by some plant species as protection that can act in animal metabolism or the growth of some ruminal microorganisms (Van Soest 1994; Brinckmann and Wollschalaeger 2003). Tannins are a group of phenylpropanoids categorized as plant defensive chemical (PDC) or plant antinutritional factors, like many other chemical structures, including saponins, gallate esters, terpenoids, flavonoids, alkaloids, coumarins, cyanides, and essential oils (Van Soest 1994; Bravo 1998). The frequent appearance of tannins is commonly divided into two classes: hydrolyzable and condensed tannins, whose structures are distinctly different. Condensed tannins (CT), or proanthocyanidins, are phenolic compounds that contain flavonoids units with high molecular weight (1000-20000 Da) that enclose ample phenolic hydroxyls and carboxyl to create a strong complex with protein, macromolecules, and polysaccharides (McLeod 1974; Hovarth 1981; Van Soest 1994). However, extensive definitions could incorrectly incorporate other compounds that behave like CT, suggesting that CT may not be classified by one definition (Tedeschi and Fox 2020).

Hydrolyzable tannins are either made of gallic or ellagic acid associated with a sugar molecule (Reed 1995), hydrolyzable tannins (HT) are broadly degraded in the rumen by ruminal bacteria, which can be likely toxic to ruminants (Holliman 1985; Shi 1988). Generally, CT are usually found in the new leaves and flowers as protection against pathogens, insects, and ruminants (Swain 1979; Van Soest 1994; Aerts et al. 1999; Álvarez Del Pino et al. 2001).

Environmental and seasonal stress such as water stress, temperatures, and light intensity can increase the tannin content of plants (Rhoades 1979; Van Soest 1994). The CT present in different plant species contain different chemical and physical properties (Mangan 1988). These are dispersed throughout the plant kingdom, commonly among trees and leguminous plants (McLeod 1974; Silanikove et al. 1994). However, not all plant species produce CT and, among those that do, concentrations and biological activity are variable. Condensed tannins are usually considered an antinutritional factor due to their ability to bind to proteins, organic compounds, metal ions, as well as reduce dry matter intake (DMI). However, most leguminous plants that contain a high concentration of CT also show high nutritional value (rich in protein), which perhaps facilitate animals that graze these legumes to neutralize their anti-nutritional effect (Naumann et al. 2017).

1.2.3.2. Condensed Tannins and Their Effect on Enteric Fermentation

Ruminal fermentation is an exclusive mechanism where end-products from the fermentation of one specie will serve as the substrate for another to keep the controlled anaerobic conditions in the rumen (Russell 2002). The association of different microorganisms is vital for the control of digestion and nutrient synthesis in the rumen (Tedeschi and Fox 2020). As previously stated, the fermentation of substrate (feed) by the rumen microbes generally produces energy for the host (VFA as a carbon source), but in addition, CO_2, hydrogen gas (H_2), and NH_3

are also released in this process. High concentrations of H_2 in the rumen can impair fermentation by obstruction of bacterial dehydrogenases and, consequently, reduce fermentable organic matter degradation, which affects VFA production and, therefore, energy been provided to the host (Tedeschi and Fox 2020). Due to this fact, reducing the hydrogen pool in the rumen is critical to keep ruminal functions.

The major hydrogen sinks in the rumen environment include methanogenic Archaea that produce methane by utilizing H_2 to reduce CO_2 (Russell and Wallace 1997). The CH_4 produced in the rumen is released by eructation to the atmosphere. Murray et al. (1976) estimated that the CH_4 produced in the rumen accounts for up to 95% of the total CH_4 eructed in sheep. As discussed previously, CH_4 emissions from domestic ruminants substantially contribute to the total CH_4 and, therefore GHG emissions, that influence global climate change (Wuebbles and Hayhoe 2002; Tedeschi et al. 2011).

Scientific and public interests have led to the search for strategies to mitigate CH_4 emissions from domestic ruminants (Janssen 2010; Tedeschi et al. 2011). Some products such as nitrate and sulfate can also consume hydrogen in the rumen and therefore reduce substrate for methanogens to produce methane (Van Zijderveld et al. 2010). However, utilization of these products in livestock production may be economically infeasible, and also may cause toxicity to the animal.

CT, as a potential strategy to mitigate enteric methane production, has been proposed by several studies (Tedeschi and Fox, 2020). Woodward et al. (2004) evaluated the effectiveness of feeding *Lotus corniculatus* (2.4-4% CT in the DM) and ryegrass hay with or without the inclusion of polyethylene glycol (PEG), as an alternative ingredient to neutralize CT binding ability, on grazing dairy cows and reported that feeding it without the addition of PEG, which

neutralizes CT, reduced CH_4 production at 13% when compared to ryegrass hay and *L. corniculatus* with PEG. The authors suggested that 65% of the reduction was due to CT. Carulla et al. (2005) compared the effectiveness of replacing ryegrass by alfalfa or red clover with supplementation of *Acacia mearsnii* extract (61.5% CT in the DM) at 0 or 41g/d in sheep diets. They noted that the addition of the CT extract provided a reduction of 13% of CH_4. Grainger et al. (2009) also evaluated the effect of three levels of inclusion of *A. mearsnii* (0, 0.9, and 1.8% of DM) extract upon methane emission of lactating dairy cows, and the authors concluded that the lower and higher dosage of CT extract reduced methane emissions by 15 and 19% respectively. However, a reduction in milk production was observed with the addition of CT.

1.2.3.3. Nitrogen Utilization

CT can depress ruminal protein degradation by reducing the rapidly degradable fraction as well as degradation rate of protein (Frutos et al. 2000; Hervas et al. 2000), which will reduce the formation of ammonia in the rumen (Barry and Manley 1984; Frutos et al. 2004). Dschaak et al. (2011) reported low ammonia concentration in the rumen and also reduced milk urea nitrogen in dairy cows when fed quebracho (*Schinopsis balansae*) CT extract at 3% of DM. The reduction of ammonia concentration in the rumen may impact FC fermenting bacteria growth, thereby reducing FC digestion. However, the lower production of ammonia in the rumen caused by the addition of CT has been reported to decrease N excretion in urine (West et al. 1993; Grainger et al. 2009), which is beneficial for the environmental (Patra and Saxena 2010), due to the fact that fecal nitrogen is less volatile than urinary nitrogen that is mainly excreted as urea, which can be easily hydrolyzed to nitrate (Misselbrook et al. 2005; Eckard et al. 2010).

CT can also affect proteolysis by inhibition of endogenous enzymatic activity in some bacteria (Oh and Hoff 1986; Horigome et al. 1988). The effect of the CT on enzymatic activity

was related to the capacity of phenolic compounds to cover the protein surface resulting in less surface area available for the enzymes to interact with the substrate (McManus et al. 1981). The minimal concentration of CT to potentially decrease proteolysis is not clear. Tanner et al. (1994) suggested that the ratio of CT: protein (g/g of protein) needed is 1:10, while Jones and Mangan (1977) proposed that the below 1:12 there was no effect on proteolysis, the possible difference found by these authors may be related to the chemical structure and molecular weight of tannins utilized. Seigler (1998) suggested that a greater degree of polymerization may negatively impact the protein-binding capacity of CT. However, the degree of binding ability of phenolic compounds such as CT is dependent on the chemical structure, molecular weight, degree of polymerization, as well as the substrate being associated (Smith et al. 2005), proteins in specifically can have different affinity for phenolics, the high molecular weight of the protein has been associated with creating a stronger complex with phenolics (Asquith and Butler 1985).

Although CT can decrease proteolysis and impact microbial protein synthesis, lower concentrations (up to 3% of DM) in ruminant diet do not impact microbial crude protein outflow in the rumen (Bhatta et al. 2000; McNeill et al. 2000; Min et al. 2003; Al-Dobaib 2009). The addition of CT up to 4% of DM as a supplement for cattle receiving low-quality tropical grass, did not compromise microbial protein synthesis or microbial crude protein (Piñeiro-Vázquez et al. 2017), whereas the highest dosage of 4% impacted voluntary feed intake. The inclusion of CT in the diet can also indirectly impact ruminal undegradable protein of the diet, once the CT-protein complex is not available for the microbes at ruminal environmental (e.g., pH) but can be disassociated in the abomasum and the duodenum, increasing the flow of protein to be absorbed as amino acids in the small intestine (SI). CT supplementation can enhance protein flow to the SI and improve the supply of metabolizable protein (MP). Supplementation of quebracho CT at

0.4% of DM in a high-concentrate diet with soybean meal as a source of protein improves MP availability by 19.5%, with no changes in intake (Mezzomo et al. 2011). An enhancement of MP supply can improve animal performance when MP is limited. Orlandi et al. (2015) also noted an increase of N retention, and efficiency of N utilization when Holstein steers received CT from *A. mearnsii*. Rivera-Méndez et al. (2017) fed Holsteins finishing steers with four different inclusions of quebracho CT (0, 0.2, 0.45, and 0.6% of DM) and noted a 6.5% increase in average daily gain when compared control to all the diets receiving tannins with a linear increase with the level of supplementation, which increased gain efficiency of the treatments receiving tannins, these results could be caused by better utilization of the protein, increasing MP utilization by the animal or by less energy been lost by protein turnover (excretion of N in the urine) (Owens et al. 2016). Similar results in cattle fed finishing diets have been reported when tannin was added either as CT, HT, or a combination of both (Barajas et al. 2010, 2011).

Owens et al. (2016), in a review of protein utilization by feedlot cattle, reported that rate of gain is linearly related to the CP concentration of the diet; however, the authors suggested that this might be caused by greater dry matter intake of diets with high CP, which increases energy intake. The authors performed a regression from a data set of 19 trials involving 43 diets with different content of CP and found that maximum gain was reached when dietary CP is 15.2% of DM, which is not typically fed to feedlot cattle (Vasconcelos and Galyean 2007). However, increase CP content in the diet can be cost-effective to feedlot operations, based on the literature data addition of CT can improve N utilization, decrease energy loss from urinary N excretion, and also increase MP supply (Mezzomo et al. 2011), increasing gain (Rivera-Méndez et al. 2017) and likewise reduce ammonia emission from the urine (Ebert et al. 2017; Koenig et al. 2018).

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2.THE EFFECTS OF SALTS OF BRANCHED-CHAIN VOLATILE FATTY ACIDS PROTECTED WITH DIFFERENT COMBINATIONS OF ENCAPSULATION MATERIALS ON RUMINAL GAS PRODUCTION DYNAMICS WHEN INCUBATED IN VITRO WITH *Brachiaria brizantha* cv. Marandu.

2.1. Overview

This study evaluated the effects of seven different encapsulations (TRT: S1 - S7) of salts of branched-chain volatile fatty acids (BCVFA), using different combinations and material sources (dioxides, maltodextrin, and celluloses), on the in vitro rumen gas production dynamics of *Brachiaria brizantha* cv. Marandu. Air-dried *B. brizantha* hay and salts of BCVFA were incubated for 48-h with fresh rumen inoculum collected from four ruminally cannulated steers. Salts were investigated at two levels of inclusion (LI; L3 = 3% and L6= 6% of DM). Six incubations were performed with (n = 3) or without (n = 3) the addition of casein and cysteine (C&C). Quantification of ivNDFD and methane (CH_4) production was performed for all samples. With C&C inclusion, S1 produced more gas than S5 and reduced lag time compared to S6 and S7. The S5, S6, and S7 had lower CH_4 production relative to S3. The L3 had higher computed TDN and ME than L6. Without C&C inclusion, there was an interaction of salt × LI on total gas production, fractional rate of gas production, lag time, gas production of fibrous and non-fibrous carbohydrate pools, CH_4 production, and energy values. The inclusion of C&C altered the fermentation parameters of salts of BCVFA with the LI of salts was dependent on N availability. The utilization of S1 salt is recommended for maximal TDN of *B. brizantha* cv. Marandu compared to other combinations of encapsulation materials.

2.2. Introduction

Animal-based products account for up to 60% of global protein consumption FAO (2006), with estimates that animal protein consumption will more than double by 2050 (National Research Council, 2015). However, the loss of agricultural lands will likely constrain the area on which livestock production occurs (Pretty et al., 2011), requiring improvements and innovations in animal nutrition to meet product demand. The vast majority of studies investigating methods of improving beef cattle production have focused on confined settings using grain-based diets (Carvalho et al., 2017). Unfortunately, this is not representative of the impending issues as approximately 70% of the world`s cattle population resides in tropical and subtropical regions (Cooke et al., 2020) that rely on the conversion of fiber into human products (Tedeschi et al., 2012). In these regions, low available crude protein (CP) is a major dietary limitation that negatively affects ruminal microbial growth and digestion (Leng, 1990). Ruminal microflora, in particular, cellulolytic bacteria, require branched-chain volatile fatty acids (BCVFA) for growth (Bryant and Doetsch, 1955; Allison and Bryant, 1963; Dehority et al., 1967) that are endogenously produced from degradation products of branched-chain amino acids (BCAA: valine, leucine, and isoleucine, respectively) (Andries et al., 1987). Hence, low dietary CP or BCAA concentrations can constrain ruminal concentrations of BCVFA, inhibiting microbial activity, and growth (Andries et al., 1987). Previous studies demonstrated that the supplementation of BCVFA increased: cellulolytic bacteria growth (Eugène et al., 2004), feed intake (Hungate and Dyer, 1956), average daily gain (Liu et al., 2016), and total ruminal volatile fatty acids (VFA) in animals consuming high-fiber diets (Liu et al., 2009).

Considering the importance of BCVFA on bacterial growth and BCAA balance in the rumen (Tedeschi et al., 2000), supplemental BCVFA products could improve the efficiency of animals grazing forages with low protein content. Unfortunately, BCVFA has to remain in the

rumen longer than the mean retention time and generation time of the bacteria that convert them into BCAA. Therefore, the objective of this study was to evaluate different encapsulations of BCVFA salts, using different combinations and material sources on the ruminal fermentation dynamics of *B. brizantha* cv. Marandu, utilizing an in vitro gas production technique (IVGP), to determine which combinations and amounts were most effective.

2.3. Materials and Methods

All experimental procedures involving animals were cared for according to guidelines approved by the Instructional Animal Care and Use Committee (AUP #2016-0362).

2.3.1. Experimental Design

Seven BCVFA salts (S1 to S7; NutriCattle, Vitoria, ES, Brazil) derived from the combination of different amounts of encapsulation materials (dioxides, maltodextrin, and celluloses) were used in this experiment. The encapsulation of the salts varied based upon the combinations and proportions of dioxides and maltodextrins (S1, S2, S4, S6, and S7) and celluloses (S3, S4, S5, and S6). The total content of BCVFA (i.e., isobutyrate, isovalerate, and 2-methylbutyrate) of each salt was similar (ranging from 81 to 95% dry matter) regardless of the encapsulation process. Salt incorporation was performed at two levels of inclusion (LI; L3 at 3% and L6 at 6% of DM).

Brachiaria brizantha cv. Marandu hay was selected as the fermentative substrate due to its relative abundance in tropical production systems. The hay sample was air-dried at 55°C for 72 h and ground to pass through a 2.0-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). A subsample (50 g) of the hay was sent to Cumberland Valley Analytical Services (Waynesboro, PA) for nutritive analyses via wet chemistry (Table A-1). The in vitro gas production technique was performed as described by Tedeschi et al. (2009), utilizing a total of

six incubations (i.e., runs). The in vitro buffering media of Goering and Van Soest (1970) was used to investigate the effect of amino-N supply; three incubations were conducted using the standard buffering media, and three were performed using media without the addition of casein and cysteine (C&C).

The incubations were performed over a span of four weeks using a single fermentation chamber capable of housing 32 Wheaton Bottles (160 mL) and containing a multiple plate stirrer to simulate rumen motility while maintaining a temperature of 39°C. Within a run, each of the seven salts was randomly assigned to four bottles, of which two received L3 and two L6, totaling 28 bottles containing BCVFA salts. The bottles were prepared prior to the in vitro incubations by weighing into the bottles 200 mg of the ground hay and the corresponding salt LI, 6 and 12 mg for L3 and L6, respectively. Next, 2.0 mL of distilled H2O and 14 mL of in vitro buffering media were added to each bottle under continual flushing of CO2 to maintain an oxygen-reduced environment. The bottles were then sealed with butyl rubber stoppers and crimp sealed and placed into the fermentation chamber to attain 39°C to mitigate microbial cold shock. Lastly, 4.0 ml of rumen inoculum was added using a syringe and needle. For each run, the fermentation chamber contained 28 bottles with hay sample and BCVFA salts, two bottles containing alfalfa (*Medicago sativa*) hay as the laboratory standard, and two blank bottles (without feed and salt)

2.3.2. Rumen Inoculum Collection

Four ruminally cannulated steers (635 ± 40 kg) were utilized as rumen fluid donors during this trial. The animals were housed in an open paddock with ad libitum access to *Cynodon dactylon* (bermudagrass) hay and clean water. Rumen inoculum (approximately 500 mL) was collected prior to each run using a rumen fluid extractor fitted with a suction-strainer. The ruminal fluid was collected from each animal and immediately placed into individual stainless-

steel thermoses to maintain temperature and maintain anaerobiosis during transportation to the laboratory. In the laboratory, under constant purging with CO2, each rumen fluid was filtered through 4 layers of cheesecloth and glass wool. Individual rumen fluids were transferred in equal portions to a pre-warmed Wheaton bottle flushed with CO2 to create a composite inoculum sample and crimp sealed.

2.3.4. In Vitro Gas Production Measurements

Before initiating data recording, the internal pressure of the bottle was equilibrated to atmospheric pressure. The pressure in the bottles was recorded at 5-minute intervals over a 48-h period, totaling 576 data points, using PicoLogo Software (Pico Technology, St Neots, United Kingdom). After the 48-h incubation period, bottles were placed in an ice bath to cease the fermentation process. All bottles had CH_4 concentrations of the headspace enumerated using gas chromatography (GOW-MAC Series 580, Gow-Mac Instrument, Bethlehem, PA) according to the method of Allison et al. (1992). The in vitro neutral detergent fiber (ivNDF) digestibility was determined by adding 40 mL of a neutral detergent solution into each bottle and autoclaving for 15 minutes at 120°C. Bottle contents were gravimetrically filtered using Whatman 54 paper and a slight vacuum. Residues were oven-dried at 55°C for 48 h and subsequently weighed after equilibrating to room temperature inside a desiccator.

2.3.5. Data Analysis

The fermentation profile and gas production kinetics were estimated through nonlinear functions using Gasfit (http://www.nutritionmodels.com/gasfit.html), and R scripts with nls and port algorithm (Fox et al. 1978; Gay 1990; Chambers and Bates 1992). Gasfit outputs were obtained for exponential and logarithmic two-pool models. Parameters obtained for the exponential curve include total gas production (mL), fermentation rate (%/h), and lag time (h).

Similarly, the log two-pool model parameters include asymptote cumulative gas production of nonfiber carbohydrate (NFC) pool (mL), fractional rate of fermentation of the NFC pool (%/h), asymptote cumulative gas production of the fiber carbohydrate (FC) pool (mL), and fractional rate of degradation of the FC pool (%/h) (Tedeschi and Fox 2020).

The total digestible nutrients (TDN) was computed according to Tedeschi and Fox (2020; p.234), assuming a fractional passage rate of 4%/h and utilizing equations 1 – 4.

$$aTDN = 0.98 \times (100 - NDF - CP - EE - Ash) + dCP + dEE + dNDF - 7 \quad \text{(Eq. 1)}$$

$$dCP = CP \times e^{-0.12 \times \left(100 \times \frac{ADIP}{CP}\right)} \quad \text{(Eq. 2)}$$

$$dEE = 2.25 \times (EE - 1) \quad \text{(Eq. 3)}$$

$$dNDF = NDF \times \left(\frac{kd}{kd+kp} + IDNDF - \frac{IDNDF \times kd}{kd+kp}\right) \quad \text{(Eq. 4)}$$

Where $ADIP$ is acid-detergent insoluble CP, % DM; $aTDN$ is apparent total digestible nutrients, % DM; CP is crude protein, % DM; EE is ether extract, % DM; $IDNDF$ is intestinal digestibility of NDF (assumed to be 0.2); kd is fractional degradation rate of NDF, %/h; kp is fractional passage rate of NDF, %/h, and NDF is neutral detergent fiber, % DM.

2.3.6. Statistical Analyses

All statistical procedures were performed using PROC GLIMMIX of SAS version 9.4 (SAS Institute Inc., Cary, NC). Variables were checked for outliers and removed when identified. Outliers were classified if their distance from the upper or lower quartile was three times greater than the interquartile range (Tukey 1977). Bottles were considered the experimental unit. Data were explored, by C&C, with the full factorial model of Salt, LI, and Salt × LI using the bottle within a run and run as random factors. The statistical significance was set at $P \leq 0.05$, and tendencies were assumed when $0.1 \geq P > 0.05$. Mean comparisons were explored using the least-square means when significance was detected.

2.4. Results

2.4.1. With the Inclusion of Casein and Cysteine.

The in vitro gas production kinetics for the incubations with C&C are shown in Table A-2. There was no interaction of salt x LI or LI effect for any gas measurements ($P = 0.169$). However, S1 (39.51 mL) and S3 (40.14 mL) produced more gas ($P = 0.012$) than S5 (35.49 mL). The lag time for the initiation of gas production was shorter ($P < 0.05$) for S1 (0.42 h) than for S6 and S7 (1.50 and 1.56, respectively). The fractional rate of exponential gas production did not differ among the salts (P = 0.998). The S3 salt had greater ($P = 0.013$) gas production of the FC pool than S7 (22.72 and 20.43, respectively). Methane production per gram of fermentable organic matter (FOM) incubated was greater ($P = 0.048$) for S3 than S5, S6, and S7 (83.15 vs. 68.16, 68.16, and 66.40 mg, respectively). The ivNDFD did not differ among the salts ($P = 0.467$) and LI ($P = 0.11$). However, the computed TDN (%) was higher ($P < 0.01$) for L3 compared to L6 (50.61 vs 48.86 %, respectively).

2.4.2. Without the Inclusion of Casein and Cysteine.

When C&C was not included, S × LI interactions were observed for total gas production ($P < 0.001$) and lag time to start of fermentation ($P < 0.001$), where S1 × L6 produced less gas and had a longer lag time compared to S1 × L3 (27.22 vs. 36.50 mL and 6.01 vs. 1.13h, respectively). The L3 inclusion rate had greater gas production for the NFC pool than L6 of salts S1 and S3 (P = 0.046), but the fractional rate of gas production on the NFC pool was greater for S1 × L6 compared to S1 × L3 and the other salts at L6 ($P < 0.001$). The gas production of the FC pool was also decreased ($P = 0.005$) for L6 versus L3 of S1 (20.57 vs. 16.69 mL), but the fractional rate of gas production of FC pool was again increased for L6 of S1 (4.28 vs. 3.01 %/h) relative to L3. The equivalent exponential digestion rate increased with L6 (9.34 %/h) compared

to L3 (5.27 %/h) of S1. There was no salt or salt x LI interaction for ivNDFD (P = 0.778); however, L3 tended (P = 0.083) to increase ivNDFD compared to L6 (58.07 vs 54.12 %, respectively). Methane (mg/g FOM) immission increased (P = 0.004) for L3 versus L6 of S1 (78.28 vs. 51.40 mg, respectively). However, L6 had higher (P = 0.004) computed TDN (%) than L3 for S1 (55.52 vs 52.45 %, respectively).

2.5. Discussion

The comparison among incubations with and without the inclusion of C&C demonstrated a reduction in TGP by up to 12% for S1, S2, S3, and S4 when C&C was removed from the media. The remaining salts (S5, S6, and S7) were reduced to a lesser degree, less than 5%. The primary cause of the observed reduction in total gas production appears to be related to the steep increase in the lag time as the lag times of S1, S2, S3, and S4 ranged from 58 – to – 88% and S5, S6, and S7 ranged from 2 – to – 28%. As expected, the exclusion of C&C also impacted CH_4 production in which S1, S2, S3, and S4 had a substantial decrease (23, 17, 20, and 20%, respectively) and S5, S6, and S7 were not greatly impacted, less than 6%. Salts containing celluloses as the only encapsulation material (S3 and S5) showed a substantial decrease in the ivNDFD, 10 and 17%, respectively. In contrast, S6, which contains dioxides, maltodextrin, and cellulose as encapsulation materials, showed a slight increase (+ 3%) in ivNDFD when C&C was removed compared with the inclusion of C&C.

A critical assumption of the IVGP fermentation is that nitrogen is not a limiting factor preventing energy spilling (Van Kessel and Russell, 1996). Our underlying assumption for the in vitro incubation without the C&C was that the N content of substrates and the rumen fluid would be enough to initiate the in vitro fermentation and that bacteria would be able to convert the BCVFA to their amino acid equivalent. Thus, a minimal amount of amino N might be needed to

stimulate the utilization of BCVFA by the ruminal microbes (Tedeschi et al. 2000). In fact, ruminant requirements for CP are established to support optimal conditions to promote bacterial growth in the rumen (Tedeschi et al. 2000; Fox and Tedeschi 2003). Therefore, the rate of microbial growth is directly correlated to the availability of N in the rumen. Because microbial protein can represent the majority of the metabolizable protein (MP) utilized by the animal (Tedeschi and Fox 2020), due to its high digestibility in the small intestine, reduced levels of amino N and BCVFA can not only impact fiber fermentation in the rumen but also restrict animal performance due to the MP supply (Tedeschi et al. 2000, 2015; Fox and Tedeschi 2003; Tedeschi and Fox 2020).

The interaction of salt × LI observed on the results with the exclusion of C&C seems to be associated with the encapsulation materials and their proportion. Although S1, S2, and S7 were encapsulated utilizing the same material (dioxides and maltodextrins), S1 was the only treatment to show an effect of LI for total gas production, lag time, equivalent exponential digestion rate, CH_4 production, and calculated TDN. At the same time, S2 and S7 had similar results with 3 or 6% of salt inclusion. Regardless, S1 × L3 increased total gas production, and decreased lag time, S1 × L6 had a higher TDN, equivalent exponential digestion, and lesser CH_4 production. This is possible because S1 likely had a greater proportion of maltodextrins than dioxides, and provided an extra source of energy to the ruminal microbes despite their apparent limitation on amino N (no C&C). Because the objective is to maximize fermentability and diminish CH_4 production, 6% inclusion of S1 seems to be more effective than 3% when N is limited in the rumen.

The observed increase in total gas production of S3 compared to S5 (both used cellulose as the encapsulation material) with the inclusion of C&C suggests that not only the encapsulation

material but the proportion utilized in the encapsulation process may affect the utilization of those BCVFA by the bacteria, confirming the previous discussion. Weigand et al. (1975) noted that isobutyrate and isovalerate had the lowest rate of VFA absorption in the rumen compared to n-butyrate, n-valerate, and propionate. Because research data on the utilization rate and absorption of BCVFA by the bacteria is limited, assumptions of the efficiency of utilization of BCVFA are still unclear and, therefore, should not be estimated to 100% (Tedeschi et al. 2000; Tedeschi and Fox 2020). Thus, the rate at which BCVFA is released and becomes available for the microbes in the rumen can alter bacterial growth, thus influencing fiber fermentation. Rumen microorganisms that digest cellulose and hemicellulose, and require BCVFA for growth (Bryant and Doetsch 1955; Allison and Bryant 1963; Dehority et al. 1967), generally exhibit slower growth compared to microorganisms that ferment non-fiber carbohydrates (Fox and Tedeschi 2003). Because BCVFA can be converted to BCAA by ruminal bacteria (Annison 1954; Slyter and Weaver 1969), the concentration and availability of BCVFA in the rumen can fluctuate and, therefore, impact FC bacteria growth (Tedeschi and Fox 2020). Although there were no effects of salt, LI, or salt × LI interaction on ivNDFD, the lag time and gas production of the FC pool might provide a good indication of the BCVFA availability to the ruminal microbes. Liu et al. (2014) supplemented three levels of isovalerate (8.4, 16.8, and 25.2 g/d) for beef steers, the authors concluded that 16.8 g/d of isovalerate was optimal for low protein diets. This suggests that when the provision of isovalerate exceeds the rate of utilization of BCVFA, there is no beneficial gain. This explanation may clarify the results observed in our study. When C&C was included, the L3 had greater TDN compared to L6 (50.61 vs. 48.86%, respectively), and consequently, increased ME (1.88 vs. 1.81 Mcal/kg, L3 and L6, respectively). Because S1 had a shorter lag time when C&C was included and the L6 had faster degradation rates of the FC pool

when C&C was not included, the utilization of S1 salt seems to yield more excellent benefits to the ruminal microbes. Nonetheless, the level of inclusion of S1 recommended for maximizing the ruminal fermentability depends on the N availability in the rumen, especially those types that prevent energy spilling (Tedeschi and Fox 2020).

2.6. Conclusion

The objective of this study was to identify optimal levels of inclusion and proportions of seven different encapsulating materials to deliver BCVFA to ruminal bacteria by evaluating the in vitro gas production kinetics when incubated with or without an external source of nitrogen (cysteine and casein). The results indicated that different materials and proportions utilized to encapsulate BCVFA impacted their utilization and altered the fermentation profile of *Brachiaria Brizantha* hay. Furthermore, the provision of cysteine and casein as a N source modified the optimum level of inclusion to maximize fermentability and lower methane production. Further research is warranted to better understand the efficiency of which bacteria are utilizing BCVFA and its impact on overall ruminal fermentability. Our study indicated that the utilization of dioxides and maltodextrins without cellulose (S1 salt) is recommended for reducing lag time for microbial digestion and maximize the total digestible nutrient of *B. brizantha* cv. Marandu compared to other combinations of encapsulation materials and proportions.

2.7. References

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3. THE INFLUENCE OF EXTENDED SUPPLEMENTATION OF QUEBRACHO EXTRACT TO BEEF STEERS CONSUMING A HAY DIET ON DIGESTION, RUMINAL, AND BLOOD PARAMETERS

3.1. Overview

Adding natural plant secondary compounds to ruminant feed has been extensively studied because of their ability to modify digestive and metabolic functions. Condensed tannin (CT) supplementation can potentially alter ruminal fermentation and mitigate methane (CH_4) emissions. The objective of this study was to determine the effect of quebracho CT extract (QT; *Schinopsis balansae*) within a roughage-based diet upon ruminal digestibility and kinetics. Twenty rumen cannulated steers were randomly assigned to four dietary treatments: QT at 0, 1, 2, and 3% of DM (QT0: 0% CT; QT1: 0.70% CT; QT2: 1.41% CT; and QT3: 2.13% CT). In situ DMD increased in a linear fashion ($P = 0.048$) as QT inclusion increased, whereas NDFD was not altered among treatments ($P = 0.980$). Neither total VFA concentration or acetate to propionate ratio differed among dietary treatments ($P = 0.470$ and $P = 0.873$, respectively). However, QT3 had lower isovalerate and isobutyrate concentrations compared to QT0 ($P \leq 0.025$), ruminal NH_3, and blood urea N tended to decline ($P \leq 0.075$) in a linear fashion as QT inclusion increased. Ruminal protozoa count was reduced in quadratic fashion ($P = 0.005$) as QT inclusion increased, where QT1 and QT2 were lower compared to QT1 and QT3. Urinary N excretion tended to reduce in a linear fashion ($P = 0.080$) as QT increased. There was a TRT × day interaction for in vitro total gas production and fractional rate of gas production ($P = 0.013$ and $P = 0.007$, respectively), and in vitro NDFD tended to be higher for QT treatments compared to no QT inclusion ($P = 0.077$). There was a TRT × day interaction ($P = 0.001$) on CH_4

production, where QT3 had the lowest compared to QT0 on Day 0 and QT2 on Days 7 and 28. Feeding QT up to 3% of the dietary DM in a roughage-based diet does not sacrifice overall DM digestibility and ruminal parameters over time, but it is not clear why QT2 did not follow the same pattern as in vitro gas parameters. Further evaluation is required to fully define CT influences on ruminal fermentation parameters and CH_4 production.

3.2. Introduction

Systematic antibiotic use increases the production and growth efficiency of livestock (Potter et al. 1976; Tedeschi et al. 2011; Huang et al. 2018). However, public apprehensions concerning the use of antibiotics and 'non-natural' products in domestic livestock diets, coupled with the livestock industry becoming a focal point of greenhouse gas (GHG) emissions, has encouraged the livestock sector to seek natural feed alternatives. Ruminants produce methane (CH_4) as one of the end products of anaerobic fermentation, by which methanogens reduce CO_2 to CH_4 as a process of eliminating H_2 from the rumen to maintain dynamic equilibrium (Janssen 2010). However, this strategy can constitute a considerable portion (2 – 10%) of the daily gross energy intake lost by the animal (Holter and Young 1992; Bodas et al. 2012). Thus, a reduction in ruminal CH_4 could result in more energy available to the animal and assist in mitigating environmental emissions (Tedeschi and Fox 2020).

Condensed tannins (CT) are plant secondary compounds that were originally categorized as antinutritional factors for large ruminants because of the observed reduction in ruminal cell well and protein digestibility (Van Soest 1994) and voluntary feed intake (Haslam 1989; Beauchemin et al. 2008). These negative effects of CT are due to their high reactivity, reducing the digestion of proteins and carbohydrates, and inhibiting enzymes (Haslam 1989). Nevertheless, CT also possess some beneficial features, such as reducing CH_4 emissions and

increasing N use efficiency (Ramírez-Restrepo and Barry 2005; Waghorn 2008; Bodas et al. 2012; Ávila et al. 2015; Orlandi et al. 2015). However, the diminished ruminal CH_4 production observed with CT provision to cattle has been strongly associated with the unintended reduction of fiber digestion in the rumen (Jouany and Morgavi 2007; Bodas et al. 2012). Therefore, the objective of this study was to evaluate the effect of differing rates of quebracho tannin extract in a hay-based diet upon blood and ruminal parameters, and in situ and in vitro ruminal apparent digestibility of beef growing steers.

3.3. Materials and Methods

This experiment was conducted from October to January 2019 at the Nutrition and Physiology Center, Texas A&M University, College Station, Texas. All experimental procedures involving animals were performed according to guidelines approved by the Instructional Animal Care and Use Committee (AUP #2018-0410).

3.3.1. Animals and Treatments

Twenty ruminally-cannulated British-crossbred steers (227 ± 19 kg) from the Texas A&M AgriLife Research Center in McGregor, TX, were utilized in a 67-d experiment. On Day -25, steers were ranked by body weight (BW) and housed in five pens (4 animals per pen) equipped with a Calan gate feed system (American Calan, Northwood, NH) and water trough. On Day 0, steers were randomly assigned to one of four dietary treatments (5 animals per treatment). Dietary treatments consisted of extract from quebracho (*Schinopsis balansae*, QT; containing 77.99% CT) SILVATEAM, San Michele Mondovi Italy) fed at 0, 1, 2, and 3% (DM basis; QT0: 0% CT; QT1: 0.70% CT; QT2: 1.41% CT; and QT3: 2.13% CT, respectively) of the diet. For all treatments, animals were fed a basal diet of bermudagrass hay (*Cynodon dactylon)* and a protein supplement (Table A-4) approximating maintenance energy and protein

requirements according to the Ruminant Nutrition System (Tedeschi and Fox, 2020). Measurements of protein precipitable phenols and protein-binding capacity were determined, as discussed by Norris et al. (2020). Quebracho extract contained 77.99% total CT (36.07%, 41.43%, and 0.49% of extractable, protein-bound, and fiber-bound CT, respectively) and 31.47% protein precipitable phenolics (PPP). The diet was offered once daily (0800 h) at 2.1% of shrunk BW (SBW), the addition of pre-weighed QT were hand-mixed into individual animal feed offered. Before each feeding, orts were collected, weighed, and a subsample stored at -20°C for chemical analyses.

3.3.1.2. Chemical Analysis

Feed samples were collected weekly for bromatological analyses and digestibility assays using in situ and in vitro procedures. A 100-g subsample of the feed and orts were shipped to Cumberland Valley Analytical Services (CVAS; Waynesboro, PA) for chemical analysis of dry matter (DM; Goering and Van Soest, 1970), neutral detergent fiber (NDF) with the addition of amylase and exclusive of ash (aNDFom; Van Soest et al., 1991), acid detergent fiber exclusive of ash (ADFom; Method# 973,18; AOAC, 2000), lignin using sulfuric acid (Goering and Van Soest, 1970), crude protein (Method# 990.03; AOAC, 2000) in a Leco FP-528 Nitrogen Combustion Analyzer (Leco Corporation, St. Joseph, MO), soluble crude protein (Krishnamoorthy et al., 1982), non-fibrous carbohydrates (NFC), fat (Method 2003.05; AOAC, 2006), starch (Hall, 2009), sugar (Dubois et al., 1956), a complete mineral panel (Method# 985.01; AOAC, 2000) in a Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT), and calculation of total digestible nutrients (TDN) and net energy using empirical equations.

3.3.2. In Vitro Gas Production Measurements

Within a treatment, rumen fluid contents from all of the animals were collected in a 1-L thermos 4-h postprandial and mixed in equal portions to form representative rumen fluid samples for the in vitro gas production technique (IVGP) as described by Tedeschi et al. (2009) and modifications discussed by Tedeschi and Fox (2020). The in vitro incubations were performed using Wheaton bottles (160 ml) incubated in two chambers with the temperature maintained at 39°C and a multiple plate stirrer with the capacity to house 24 bottles in each. Prior to the in vitro incubations, the Wheaton bottles were prepared using the procedure discussed by Crossland et al. (2018), but briefly: 200mg of 48h air-dried feed sample ground through a 2-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) was weighed and transferred into 40 bottles (8 replicates per treatment). Then, 2.0 mL of distilled H_2O was used to dampen the sample to prevent particle scattering, and 14 ml of Goering and Van Soest (1970) in vitro buffering media was added under continual flushing of CO_2 to maintain an oxygen-reduced environment. The bottles were sealed using butyl rubber septa and crimp seals and transferred to the 39°C chamber to attain ruminal temperature prior to rumen inoculum placement. Rumen fluids were filtered through 4 layers of cheesecloth and glass wool and transferred into a glass flask flushed with CO_2. The addition of 4 mL of rumen inoculum per bottle was then performed via syringe and needle insertion. For an individual run, each chamber contained six bottles per treatment: four bottles containing feed sample, one laboratory standard, and one blank (without the addition of feedstuff). The internal pressure of the chamber was equilibrated to atmospheric pressure prior to the initiation of data recording. Upon initiation, the pressure inside each of the bottles was recorded at 5-min intervals for 48 h using PicoLogo software (Pico Technology, Tyler, TX 75702) with real-time fermentation profiles being plotted for each bottle. After the 48-h incubation period, bottles were placed in an ice bath to cease fermentation. Methane

concentration was measured by taking a subsample of the headspace (1 mL) and analyzing via gas chromatography (GOW-MAC Series 580, Gow-Mac Instrument, Bethlehem, PA) according to the method of Allison et al. (1992). The in vitro NDF digestibility was calculated by adding 40 mL of neutral detergent solution (ANKOM Technology, Macedon, NY) in each bottle, autoclaving them for 15 min at 120°C, filtering the samples using Whatman 54 papers, and oven-drying the residue at 55°C for 48 h.

Nonlinear functions were used to plot the kinetic analysis of the 48-h fermentation, where the lowest sum of square errors (Schofield et al. 1994) was utilized to select the functions using Gasfit (http://www.nutritionmodels.com/gasfit.html). The convergence of gas production data was performed with specific R scripts using *nls* and *port* algorithm (Fox et al. 1978; Gay 1990; Chambers and Bates 1992). The Gasfit output data (Table A-7) included total gas production (mL), fermentation rate (1/h), lag time (h) using exponential curves; asymptote cumulative gas production of NFC pool (mL), fractional rate of fermentation of the NFC pool (1/h), asymptote cumulative gas production of the FC pool (mL), and fractional rate of degradation of the FC pool (1/h) using the logarithmic two-pool nonlinear function as described by (Tedeschi and Fox 2020). The IVGP dynamics were evaluated on days 0, 7, 14, 21, and 28 of the experimental period.

3.3.3. Ruminal In Situ Digestibility Measurements

Nylon bags (5 x 10 cm) with 50 μm porosity (ANKOM Technologies, Macedon, NY, USA) were utilized to perform the 48-h in situ incubations. Bags were weighed, and filled with 5 g of 2-mm oven-dried ground diet sample, and sealed as described by Vanzant et al. (1998). Representative feed samples were collected weekly and dried at 55°C for at least 48 h prior to grinding. Bags containing feed samples were replicated five times, whereas blank bags (bags

without feedstuff) were replicated twice. Feed and blank bags were placed within 32 x 42 cm polyester bags and placed in the rumen of each animal on d 0, 7, 14, 21, and 27. Blank bags were used as correction factors for the in situ digestibility of DM and NDF. Following the 48-h incubation, bags were removed from the rumen and immediately quenched in ice water to stop fermentation. The rinsing process of the bags was performed by washing the bags with distilled water followed by cold water and the spin portion of the delicate wash cycle in a washing machine (Krizsan and Huhtanen 2013).

After washing, all bags were dried at 55°C for 72 h in a forced-air oven, equilibrated to room temperature in a desiccator, and final dry weight was recorded. In situ dry matter digestibility (DMD) was calculated using the following equation:

$$DMD, \% = \frac{100 \times (W3 - (W1 \times C1))}{W2} \quad \text{(Eq.1)}$$

where W3 is the dried bag weight containing sample residue (g), W1 is initial bag weight (g), C1 is the blank bag correction factor of dried bag weight (g), and W2 is initial dried sample weight (g).

The in situ NDF digestibility was obtained by the method described by Van Soest and Robertson (1980), using the ANKOM 200 fiber analyzer (ANKOM Technology, Meadon, NY) with the addition of amylase. After washing with neutral detergent, all bags were dried at 55°C for 72 h in a forced-air oven, equilibrated to room temperature in a desiccator, and final dry weight was recorded. In situ NDF digestibility (NDFD) was calculated using the following equation:

$$NDFD, \% = \frac{100 \times (W4 - (W1 \times C2))}{W2} \quad \text{(Eq.2)}$$

where W4 is the dried bag weight containing sample residue after washing with ND (g), W1 is initial bag weight (g), C2 is the blank bag correction factor after washing with ND, and W2 is the initial weight of the dried samples (g).

3.3.4. Rumen Sampling and Analysis

During the in situ bag removal process, about 600 mL of rumen fluid was collected from each steer using a vacuum pump, and placed into individual stainless-steel thermoses. Measurements of pH and redox potential were taken, using a portable pH and redox meter probe (Thermo Scientific Orion A221, Thermo Fisher Scientific, Waltham, MA), immediately after the collection of each animal's rumen fluid. The rumen fluid was then filtered through 8 layers of cheesecloth, and subsamples were taken into duplicate falcon tubes containers for the preservation of volatile fatty acids (VFA) and NH_3-N analyzes, and protozoa enumeration.

Preservation methods and analyses were performed, as described by Norris et al. (2020). Briefly, preservation methods for analyses were: 8 mL of rumen inoculum and 2 mL of metaphosphoric acid solution (25% w/v) for VFA, 2 mL of rumen inoculum and 8 mL of 0.1*M* HCl acid solution for NH_3-N analyses, and 1 mL of rumen inoculum and 10 mL of ethanol for protozoa enumeration. All samples were stored at -20°C until subsequent analyses. The concentrations of VFA was determined using gas chromatography as described by Cagle et al. (2019) and NH_3-N via colorimetric methods. Protozoa counts were obtained by methods described by Dehority (1984) without staining. The protozoa counting technique was performed using a Sedgewick Rafter counting chamber, 1-mL aliquot of the diluted sample (1:10 rumen fluid: ethanol) were counted at 100x magnification with a 0.5-mm square counting grid, 25 evenly spaced grids from the entire chamber surface were counted using Nikon Eclipse E200 microscope (Nikon Corporation, Tokyo, Japan).

3.3.5. Blood Sampling and Analysis

On d -1, 6, 13, 20, and 27 blood samples were collected via jugular venipuncture into sodium heparin blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) and immediately placed on ice. Plasma was obtained by centrifuging the blood (3,000 x g for 20 min at 4°C) and aliquoting the supernatant into polypropylene tubes, and samples were stored at -20°C until analysis. Samples analyzed for blood urea nitrogen (BUN) using a colorimetric kit (#B7551, Pointe Scientific, Inc., Canton, MI). The intra- and inter-assay coefficient of variation was 3.0% and 5.8 %, respectively. Urinary N excreted (UNE) was then estimated using the equation of Kohn et al. (2005), $\mathrm{UNE} = \mathrm{CR} \times \mathrm{BUN} \times \mathrm{BW}$, where CR is clearance rate of N in the kidney of beef cows (1.3 L × d^{-1} × kg^{-1}), BW is the body weight (kg), and BUN is blood urea N (g/L).

3.3.6. Statistical Analysis

All data were analyzed using the PROC MIXED of SAS version 9.4 (SAS Institute Inc., Cary, NC). The Shapiro-Wilk test from the UNIVARIATE procedure was performed on all dependent variable data sets to check for normality, and ruminal protozoa count was not normally distributed ($W = 0.86$). Thus, ruminal protozoa count was log-base 10 transformed to achieve normality ($W = 0.97$). All data were analyzed in accordance with a repeated measures design using the REPEATED statement. For each variable, the covariance structure was determined according to the Akaike information criterion. The model for the in vitro data was bottle nested with treatment and chamber as an experimental unit, and box as a random variable. All other variables were examined, having pen and animal nested within treatment as a random factor. The specified term for the repeated statement was day, with the animal within treatment or bottle within treatment and chamber as the subject. Dry matter intake was included as a

covariate for the in situ and ruminal parameter data. For blood parameters, blood obtained prior to the initiation of the trial (d -1) was included as an independent covariate within the analyses. Least square means were determined using the LSMEANS statement, the largest standard error of the mean is reported. Results are reported according to treatment effects if no interactions were significant. Orthogonal polynomial contrasts were performed for all variables to determine linear, quadratic, and control vs. QT effects. Significance was set as $P \leq 0.05$, and tendencies were assumed as $0.1 \geq P > 0.05$.

3.4. Results

Table A-5 shows the effect of QT inclusion on the intake and in situ data. Final SBW and ADG did not differ among treatments ($P = 0.698$ and $P = 0.795$, respectively). As all treatments received the basal diet at approximately maintenance level (2.1% of SBW), the lack of difference in ADG and FSBW was expected. There was no effect of QT inclusion upon DM or NDF intake ($P = 0.647$ and $P = 0.641$, respectively). In situ DMD increased in a linear fashion with the inclusion of QT ($P = 0.048$) with a significant DMI × treatment effect ($P = 0.044$), Figure B-7 illustrates the effect of different DMI levels on DMD. Increased DMD was observed for QT3 ($P = 0.019$) compared to QT0 at a low level of DMI. As DMI increased, in situ DMD also increased for all treatments. However, DMD of QT3 only increased 1.25% from the low (3.25 kg/d) to high (5.5 kg/d) intake, whereas QT0 and QT2 increased 11%, on average, and QT1 increased 5.45%. It is not understood why QT2 trended differently than QT3 and QT1; however, this same trend has occurred previously for the intermediate level of QT inclusion (Norris et al. 2019 and 2020). In contrast to DMD, there was only a day effect for NDFD ($P < 0.001$) with DMI having a tendency ($P = 0.058$). There were no treatment effects for ruminal pH, reactive oxygen species

(ROS), and mV ($P = 0.520$). However, there was a day effect ($P < 0.001$) on pH and ROS values.

Overall, the inclusion of QT did not greatly influence ruminal parameters (Table A-6). Isobutyrate concentration increased ($P = 0.009$) for QT0 relative to QT2 and QT3 (0.84 vs 0.77 and 0.73 mmol/L, respectively) with the inclusion of QT resulting in less isobutyrate than without QT ($P = 0.009$; 0.76 vs 0.84 mmol/L). Similarly, isovalerate was greater without QT versus with QT provision ($P = 0.040$; 0.86 vs. 0.78). A linear tendency ($P = 0.074$) to decrease ruminal NH3-N concentration was observed as QT inclusion increased, and the same pattern was observed on BUN and UNE values ($P = 0.079$ and 0.080, respectively). Ruminal protozoa count was affected in a quadratic fashion ($P = 0.005$) as QT inclusion increased, with QT1 and QT2 being lower relative to QT0 and QT3 (4.98 vs. 5.06 log10/mL, respectively).

In vitro gas production dynamics are shown in Table A-7. Total gas production decreased in a linear fashion ($P = 0.051$) as QT inclusion increased. However, there was an interaction of TRT × Day (Figure B-8) for total gas production ($P = 0.013$) and the fractional rate of gas production ($P = 0.007$). The asymptote cumulative gas production of the NFC pool (P1) was affected by TRT × Day ($P = 0.015$), but there were no effects for the fractional rate of gas production of the NFC pool ($P \geq 0.038$). Neither lag time required to commence fermentation nor time required to start the asymptote of the FC pool was altered by TRT or TRT × Day interaction ($P \geq 0.186$). There were no effects for the asymptote of the FC pool ($P \geq 0.260$). However, there was a TRT × Day interaction for the fractional rate of gas production of the FC pool and exponential degradation rate ($P = 0.039$ and $P = 0.043$). In vitro NDFD increased in a linear fashion ($P = 0.009$) as QT inclusion increased. This resulted in the cumulative gas production (CGP) per gram of NDFD to be reduced ($P = 0.027$) for QT3 relative to QT0 and the

inclusion of QT to have lesser CGP compared to without QT (P = 0.033; 293.8 vs. 364.8 ml/g NDFD). The computed TDN and ME was affected by a TRT x Day interaction ($P \leq 0.001$); however, the QT treatment showed higher ($P < 0.001$) TDN and ME compared to no QT.

3.5. Discussion

The lack of QT inclusion effects upon intake and ADG is similar to the results showed by Beauchemin et al. (2007), when QT extract was included up to 2% of the dietary DM. Similarly, QT inclusion up to 4.5% of DMI did not affect DM, OM, or NDF intake in beef cattle limit fed near maintenance requirements (Norris et al. 2020a, 2020b). Overall, previous studies evaluating the effects of CT on feed intake have shown variable results. Dschaak et al. (2011) observed reduced DMI in lactating cows when QT was included at 3% of dietary DM. Whereas, Woodward et al. (2001) reported an increase in DMI when fed *Lotus pedunculatus* (2.59 % of CT) to cows in late lactation. The effects of CT on intake seem to be more pronounced when the inclusion exceeds 5% of the diet DM (Frutos et al., 2004; Naumann et al., 2017). Many species that contain CT also produce other plant secondary compounds; therefore, the alteration in DMI cannot be solely attributed to CT when only CT is measured as other metabolites may also be responsible for the observed variation (Naumann et al. 2017).

In the current study, the inclusion of QT in the diet enhanced in situ DMD in a linear fashion. However, this observation contradicts other reports (Orlandi et al. 2015; Piñeiro-Vázquez et al. 2017; Norris et al. 2020a). Landau et al. (2000) demonstrated that the ruminal passage rate could be reduced when animals consume CT. An increase in ruminal retention time will directly influence overall microbial digestion. Although in situ DMD improved in all the dietary treatments as DMI increased, QT3 displayed a reduced slope relative to all other treatments. Nevertheless, the current results are plausible since the inherent dynamics associated

with passage rate, and post-ruminal digestion are not represented as the majority of the studies observing reduced DMD with QT inclusion evaluated total tract not solely ruminal digestibility. Whereas, the relatively neutral ruminal pH values in the current study were expected due to the high fiber content of the basal diet.

The lack of treatment effect on total VFA and the primary VFAs (acetate, propionate, and butyrate) indicates that ruminal digestibility was most likely not affected by QT inclusion in the diet. This is consistent with previous results feeding QT (Aguerre et al. 2016). To a degree, this confirms the in situ NDF digestibility and ruminal pH values observed. However, contrary to our results, Beauchemin et al. (2007) noted a linear reduction in total VFA and acetate production with 1 and 2% QT inclusion, but neither total tract DM or NDF digestibility were altered compared to the control group. Condensed tannins have displayed the ability to reduce ruminal protein degradation (Frutos et al. 2000; Hervas et al. 2000), reducing the production of NH_3 in the rumen. This is consistent with the current study as ruminal NH_3 was reduced in a linear fashion as QT increased. Similarly, other studies supplementing QT in a high roughage diet have observed reductions in ruminal NH_3 concentration. (Beauchemin et al. 2007; Norris et al. 2020b, 2020a) Since isobutyrate and isovalerate are primarily derived from the deamination of branched-chain amino acids in the rumen (Cummins and Papas 1985), the decrease in isobutyrate and isovalerate concentration as QT inclusion increased is indicative of reduced ruminal protein degradation, corroborating the ruminal NH_3 and BUN values in the present study. However, the BUN levels observed (19.65 – 23.00 mg/dl) greatly exceed the optimum level (5 to 8 mg/dl) proposed by Johnson and Preston (1995). This may suggest excess N intake in the current experiment but is likely just representative of asynchrony between the degradation rates of protein and carbohydrates (Tedeschi and Fox 2020). The linear decrease in UNE as QT

inclusion increased suggests a shift in N excretion from the urine to the feces. Previous research has demonstrated the potential for QT to modify the route of N excretion from the urine to the feces (Orlandi et al. 2015; Aguerre et al. 2016; Norris et al. 2020b); this is assumed to be a result of reduced ruminal proteolysis.

The quadratic effect of CT on ruminal protozoa has been reported in studies utilizing QT (Norris et al. 2020b) and *Leucaena leucocephala* (Tan et al. 2011). By contrast, Jolazadeh et al. (2015) reported a linear decrease in the protozoa population when evaluating the effects of soybean meal treated with CT from pistachio extract, and Chiquette et al. (1989) showed an increase in ruminal protozoa population when sheep were fed CT from *L. corniculatus*. However, the mechanism by which CT alter rumen dynamics and protozoa population is not well understood (Patra and Saxena 2010).

The results of the in vitro gas production dynamics demonstrated a slightly different pattern from those obtained using the in situ technique. The reduction in gas production of the NFC pool on Day 0 for QT3 may suggest a reduction in the rapidly degradable fraction of the feedstuff, in particularly the dietary protein (Frutos et al. 2000). However, the gas production of QT3 was similar to all other treatments on Days 7 and 14. This could indicate an adaptative response by the ruminal microbes (Chiquette et al. 1989; McSweeney et al. 2001). The tendency for QT inclusion to increase the rate of gas production of the NFC pool indicates that QT may reduce the total digestion of the rapidly degradable fraction, but the rate in which it is digested is equal to or greater than the control treatment.

The effects of feeding CT-rich plants or CT extract to ruminants have demonstrated favorable results upon CH_4 emissions (Tedeschi et al. 2014). The treatment × day interaction for CH_4 production likely indicates inhibition of the methanogenic bacteria in the rumen on Day -0

of QT feeding, but ruminal adaptation may have occurred as no treatment differences were detected on days 14 and 21. On day 28, QT3 had the least CH_4 production per gram of NDF digested with QT2 having elevated CH_4 production relative to all other treatments. Tavendale et al. (2005) proposed two mechanisms to explain the possible inhibition effect of CT on CH_4 production, either by reducing fiber digestion, thereby decreasing the H_2 pool in the rumen, or by directly inhibiting methanogenic bacterial growth. However, in the current study ivNDFD was not affected by QT inclusion, which suggests that the decreased CH_4 production demonstrated in steers fed QT3 on days 0 and 28 may be a result of a lower methanogen population in the rumen.

In research settings, the effects of QT upon CH_4 emissions have been variable. Beauchemin et al. (2007) did not observe a difference in CH_4 production when QT was fed up to 2% of dietary DM. By contrast, Norris et al. (2020a) reported a linear reduction in CH_4 production when QT was fed at 1.5, 3, and 4.5% of DM, yet the daily emission was only reduced at the highest dosage. It appears that the efficacy of QT for CH_4 suppression is dependent upon CT amount ingested by the animal, indicating that the inclusion of QT extract up to 3% (2.13% CT) of the dietary DM might be below the threshold required to diminish CH_4 production in cattle. However, the provision of QT has demonstrated the potential to reduce not only CH_4 production but also CO_2 in cattle (Norris et al. 2020a). This may partly explain the drastic reduction in cumulative gas production per gram of NDF digested in our study.

3.6. Conclusion

Ruminal parameters were not detrimentally impacted by a provision of QT in a high roughage diet. However, in our study, the provision of QT enhanced in situ DMD for animals with low DMI, which indicates that the provision of the highest dosage modified the rumen bacteria population. It is not clear why QT2 did not follow the same pattern of QT3 and QT1. As

DMI passage rate increases and thus DMD is affected, the provision of QT seems to be beneficial for those animals with low feed intake, and higher passage rate. IVGP data indicated that the accumulative gas production of the NFC pool was reduced with QT inclusion. However, the rate of gas production tended to increase on treatments consuming QT compared to QT0, which suggests that QT inhibit the degradation of some of NFC, but what was available for the microbes was digested at the same rate or faster than the control treatment. When evaluating the overall response on digestion of QT inclusion, it appears that the ability of QT to bind to protein and carbohydrate does not turn it completely unavailable for microbial digestion, but requires a longer period for the microbes to degrade it. Thus, the provision of QT up to 3% of dietary DM can be beneficial when DMI is compromised due to quality in the forage.

Feeding QT greatly impacted ruminal NH_3 concentrations and BUN, reducing urinary N excretion. The decrease in urinary N excretion is beneficial for soil nitrogen status since urinary N is positively correlated with the production of nitrous oxide (N_2O), a potent greenhouse gas (Eckard et al., 2010). The inconsistent results for methane production in the present study indicate that the possible beneficial effect of QT on methane emissions may be dependent on nutritional factors in the diet as well as the digestion profile of the diet. Some research has evaluated CT to protein ratio and its effects on ruminal proteolysis (Jones and Mangan 1977; Tanner et al. 1994). However, the optimal dosage of CT required to diminish CH_4 production, and the impacts of fiber and energy content in the diet need to be evaluated. The effects of different molecular weight and chemical structure appears to affect not only protein-binding capacity but also CH_4 inhibition. Thus, further research is necessary to better understand the feasibility of QT utilization upon ruminal CH_4 production. In summary, our results demonstrate

that the inclusion of QT up to 3% did not impact ruminal parameters and digestion of DM and NDF in growing animals receiving a high roughage diet.

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4.SUMMARY

Different materials and proportions utilized to encapsulate BCVFA salts impacted their utilization and altered fermentation profile. However, the efficiency of which bacteria are using BCVFA and its impact on overall ruminal fermentability needs further research to better understand the bacteria's nutritional requirements and optimal level of provision of BCVFA to the animal. The utilization of dioxides and maltodextrins without cellulose (S1 salt) encapsulation material seems to be recommended to enhance microbial digestion and optimize the total digestible nutrient of *Brachiaria brizantha* cv. Marandu.

Quebracho CT extract inclusion does not detrimentally affect the overall ruminal parameters of steers consuming a high-roughage diet. However, ruminal deamination seems to be impaired with the addition of QT. The inclusion of QT diminished isovalerate and isobutyrate ruminal concentrations. Overall in situ DM and NDF digestibilities were not impacted with the inclusion of QT. Nevertheless, in vitro data showed a greater ivNDFD with QT inclusion in the diet. The utilization of QT extract to diminish CH_4 production seems to be related to the dosage of provision, the optimal dosage to reduce CH_4 appears to be above 3%. At the same time, the treatment QT1 had similar ruminal parameters with higher TDN and ME in vitro values. Making QT1 the best prospect for improving animal efficiency.

The utilization of natural feed additives such as QT extract and BCVFA can potentially benefit the efficiency of nutrient utilization of animals consuming high-roughage diets, which can reduce overall greenhouse gas emissions either by direct inhibition of CH_4 production in the rumen or by increasing efficiency of the animal, reducing the intake requirement and total lifetime to achieve the desired body weight.

APPENDIX A

TABLES

Table A-1. Chemical composition of *Brachiaria brizantha* cv. Marandu

Chemical composition[1]	%
DM, % as fed	95.5
CP, % DM	13.0
Soluble protein, % DM	5
ADIN, % DM	0.62
NDIN, % DM	2.19
ADF, % DM	36.1
NDF, % DM	63.5
Lignin, % DM	4.26
Sugar, % DM	3.9
Starch, % DM	0.9
Fat, % DM	2.02
Ash, % DM	10.48
TDN, % DM	56.9
NE_m, Mcal/kg	1.17
NE_g, Mcal/kg	0.59

[1] DM = dry matter; CP = crude protein; ADIN = acid detergent insoluble nitrogen; NDIN = neutral detergent insoluble nitrogen; ADF = acid detergent fiber; NDF = neutral detergent fiber; TDN = total digestible nutrient; NE_m = net energy for maintenance; NE_g = net energy for gain; Chemical analyses and the calculation of TDN, NEm, and NEg were conducted by Cumberland Valley Analytical Services (Waynesboro, PA).

Table A-2. In vitro gas production dynamics of incubations performed with the inclusion of casein and cysteine

	Salt[1]								LI			*P*-value[2]		
Items[3]	S1	S2	S3	S4	S5	S6	S7	SEM	3%	6%	SEM	S	LI	S × LI
Exponential														
TGP, ml	39.51^{a}	38.81^{ab}	40.14^{a}	37.76^{ab}	35.49^{b}	36.26^{ab}	36.10^{ab}	1.95	37.70	37.49	1.67	0.012	0.547	0.677
kd, %/h	5.31	5.37	5.41	5.41	5.29	5.44	5.24	0.49	5.27	5.44	0.45	0.998	0.267	0.592
Lag time, h	0.42^{b}	0.79^{ab}	0.98^{ab}	0.65^{ab}	1.29^{ab}	1.50^{a}	1.56^{a}	1.63	0.85	1.04	1.50	0.020	0.370	0.377
Log. Two-Pool														
Asymptote (P1), ml	14.36	13.94	14.05	13.50	12.66	13.06	12.66	0.67	13.45	13.47	0.34	0.327	0.961	0.684
kd P1 (%/h)	8.66	9.07	9.50	9.67	9.22	8.93	9.41	2.96	8.83	9.58	1.08	0.955	0.169	0.655
Lag time to P2, h	1.53	1.94	2.43	2.43	2.99	3.03	2.62	1.22	2.43	2.41	1.26	0.338	0.954	0.813
Asymptote (P2), ml	22.28^{ab}	22.02^{ab}	22.72^{a}	21.58^{ab}	20.90^{ab}	20.58^{ab}	20.43^{b}	1.48	21.4	21.6	1.40	0.013	0.607	0.679
kd P2, %/h	2.83	2.90	2.97	2.93	3.00	2.95	2.94	0.24	2.92	2.95	0.22	0.962	0.722	0.691
Exp. kd equivalent, %/h	4.84	5.02	5.19	5.12	5.02	5.17	5.16	0.47	4.99	5.15	0.42	0.945	0.376	0.513
Energy Estimates														
ivNDFD, 48h	60.24	58.89	62.34	55.21	61.87	53.55	58.17	5.50	60.69	56.53	4.56	0.467	0.110	0.936
Methane (mg/g FOM)	79.53^{ab}	80.59^{ab}	83.16^{a}	77.03^{ab}	68.16^{bc}	66.41^{c}	68.17^{bc}	8.23	72.96	76.48	6.94	0.048	0.313	0.861
TDN, %	49.36	49.64	49.89	49.78	49.99	49.75	49.74	0.69	50.61^{a}	48.86^{b}	0.52	0.984	0.001	0.698
ME, Mcal/kg (TDN, 4%)	1.83	1.84	1.85	1.85	1.85	1.84	1.84	0.04	1.88^{a}	1.81^{b}	0.03	0.986	0.001	0.723

[a-c] Least Squares means in a row with different superscripts differ at $P \leq 0.05$.

[1]Salts values are given as LSM.

[2] S = salt; IL = Inclusion Level

[3]TGP = Total gas production of the exponential nonlinear function; kd = the fractional rate of gas production of the exponential nonlinear function; Lag time = time required to commence fermentation; Asymptote (P1) = accumulative gas production of nonfiber carbohydrate pool (NFC); kd (P1) = fractional rate of gas production of NFC pool; Lag time to P2 = time required to commence fermentation of fiber carbohydrate (FC) pool; Asymptote (P2) = accumulative gas production of FC pool; kd P2 = fractional rate of gas production of FC pool; Exp. kd equivalent = exponential decay digestion rate (kd); ivNDFD = in vitro neutral detergent fiber digestibility; TDN = computed TDN; ME = metabolizable energy.

Table A-3. In vitro gas production dynamics of incubations performed without the inclusion of casein and cysteine

	Salt[1]								LI			*P*-value[2]		
Items[3]	S1	S2	S3	S4	S5	S6	S7	SEM	3%	6%	SEM	S	LI	S × LI
Exponential														
TGP, ml	31.86	34.42	34.02	33.75	34.46	34.54	34.75	1.49	35.24^{a}	32.70^{b}	1.33	0.214	†	†
kd, %/h	6.01	5.87	5.83	5.85	5.42	5.39	5.39	0.52	5.72	5.64	0.48	0.325	0.623	0.036
Lag time, h	3.58^{a}	2.09^{ab}	2.32^{ab}	2.24^{ab}	1.80^{ab}	1.58^{b}	1.58^{b}	0.84	1.57^{b}	2.77^{a}	0.77	0.020	†	†
Log. Two-Pool														
Asymptote (P1), ml	10.50^{b}	12.30^{ab}	12.03^{ab}	11.47^{ab}	12.39^{ab}	12.85^{a}	12.13^{ab}	0.57	13.08^{a}	10.82^{b}	0.41	0.023	†	0.046
kd P1 (%/h)	17.13^{a}	11.32^{b}	12.13^{b}	11.73^{b}	9.89^{b}	9.94^{b}	10.68^{b}	1.79	10.30^{b}	13.36^{a}	1.64	†	†	†
Lag time to P2, h	5.75^{a}	3.89^{b}	4.30^{ab}	4.14^{ab}	3.70^{b}	3.61^{b}	3.45^{b}	1.00	3.32^{b}	4.92^{a}	0.91	0.018	†	†
Asymptote (P2), ml	18.64	19.45	19.43	19.6	19.26	18.64	19.42	0.66	19.53	18.88	0.51	0.588	0.062	0.006
kd P2, %/h	3.65^{a}	3.25^{ab}	3.27^{ab}	3.29^{ab}	3.03^{b}	2.93^{b}	3.04^{b}	0.21	3.09^{b}	3.33^{a}	0.19	0.001	0.004	†
Exp. kd equivalent, %/h	7.31^{a}	5.92^{b}	5.97^{b}	5.94^{b}	5.37^{b}	5.15^{b}	5.42^{b}	0.5	5.48^{b}	6.26^{a}	0.45	†	†	†
Energy Estimates														
ivNDFD, 48h	59.79	57.16	56.72	54.05	52.96	55.09	56.9	3.08	58.07	54.12	1.62	0.781	0.083	0.779
Methane (mg/g FOM)	64.85	69.06	69.27	64.26	65.9	62.89	65.24	7.02	70.16^{a}	61.68^{b}	6.48	0.483	†	0.020
TDN, %	53.99^{a}	52.43^{ab}	52.57^{ab}	52.63^{ab}	51.70^{b}	51.05^{b}	51.85^{ab}	0.97	52.73^{a}	51.90^{b}	0.85	0.006	0.023	0.005
ME, Mcal/kg (TDN, 4%)	1.95^{a}	1.90^{b}	1.90^{b}	1.90^{ab}	1.87^{bc}	1.85^{c}	1.87^{bc}	0.03	1.91^{a}	1.88^{b}	0.03	0.005	0.023	0.004

$^{a\text{-}c}$ Least Squares means in a row with different superscripts differ at $P \leq 0.05$. Salt values are given as LSM.

[2] S = salt; IL = Inclusion Level; † = $P < 0.001$

[3]TGP = Total gas production of the exponential nonlinear function; kd = the fractional rate of gas production of the exponential nonlinear function; Lag time = time required to commence fermentation; Asymptote (P1) = accumulative gas production of nonfiber carbohydrate pool (NFC); kd (P1) = fractional rate of gas production of NFC pool; Lag time to P2 = time required to commence fermentation of fiber carbohydrate (FC) pool; Asymptote (P2) = accumulative gas production of FC pool; kd P2 = fractional rate of gas production of FC pool; Exp. kd equivalent = exponential decay digestion rate (kd); ivNDFD = in vitro neutral detergent fiber digestibility; TDN = computed TDN; ME = metabolizable energy.

Table A-4. Ingredient and chemical composition of the basal diet

Items[1]	Basal diet, %
Ingredient composition, % DM	
Bermudagrass hay	87.90
Cottonseed Meal	6.60
Dried Distillers Grain	4.41
Molasses	1.09
Chemical composition[2]	
DM, %	91.13
CP, % DM	13.18
Soluble Protein, % CP	24.45
aNDF, % DM	60.97
ADF, % DM	37.59
Lignin, % DM	10.12
Crude Fat, % DM	1.89
Sugar, % DM	6.58
Starch, % DM	1.79
NFC, % DM	15.19
Ash, % DM	8.76
Calcium	0.23
Phosphorus	0.19
TDN, %	55.15
ME, Mcal/kg	1.99
NEm, Mcal/kg	1.14
NEg, Mcal/kg	0.58

[1]Items are feed ingredients and chemical composition of diets evaluates by Cumberland Valley Analytical Services (Waynesboro, PA)

[2]DM, dry matter; CP, crude protein; aNDF, neutral detergent fiber with amylase and sodium sulfate; ADF, acid detergent fiber; TDN, total digestible nutrients; NEm, net energy for maintenance; NEg , net energy for gain;·

Table A-5. Effect of quebracho tannin extract on intake, in situ digestibility and ruminal parameters

	Dietary Treatment[1]					*P – values*			*Covariate*[2]		*Contrasts*[3]		
Item	QT0	QT1	QT2	QT3	SEM	TRT	Day (D)	TRT x Day	DMI	TRT x DMI	L	Q	QT
ISBW, kg	234.5	233.0	224.9	232.5	20.04	0.670	-	-	-	-	0.601	0.458	0.536
FSBW, kg	281.6	284.3	267.1	282.6	23.04	0.698	-	-	-	-	0.782	0.581	0.786
ADG, kg/d	0.77	0.84	0.69	0.82	0.11	0.795	-	-	-	-	0.992	0.783	0.935
DMI, kg/d	5.01	5.02	4.76	4.95	0.41	0.647	-	-	-	-	0.549	0.595	0.606
NDFI, kg/d	3.05	3.06	2.90	3.02	0.25	0.641	-	-	-	-	0.548	0.588	0.602
DMD, %	58.19	58.53	58.51	58.38	0.08	0.068	< 0.001	0.201	0.012	0.044	0.048	0.669	0.093
NDFD, %	44.96	45.19	44.65	44.93	1.13	0.980	< 0.001	0.231	0.058	0.116	0.881	0.978	0.975
pH	6.44	6.50	6.50	6.49	0.05	0.833	< 0.001	0.866	0.830	0.742	0.550	0.507	0.375
ROS	-185.27	-141.49	-164.99	-154.13	23.07	0.521	< 0.001	0.556	0.261	0.529	0.467	0.444	0.208
mV	-208.84	-222.95	-248.90	-210.34	19.27	0.356	0.577	0.931	0.117	0.326	0.243	0.707	0.169

[1]Dietary treatment values are given as least-squares means.
[2]If there were significant ($P \leq 0.05$) interactions between dietary treatment, day, and DMI the dietary treatment means are reported with the covariate structure in the model.
[3]Contrasts: L = Linear; Q = quadratic; QT = no Quebracho vs. Quebracho inclusion

Table A-6. Effect of quebracho tannin extract within a high-roughage diet on rumen and blood parameters.

	Dietary Treatment[1]					*P* - Value		Covariate[2]		Contrasts[3]		
Item	QT0	QT1	QT2	QT3	SEM	TRT	TRT x Day	DMI	DMI x TRT	L	Q	C x QT
Total VFA, mmol/L	76.08	72.86	68.70	68.64	3.83	0.470	0.325	0.117	0.135	0.141	0.685	0.192
Acetate, mmol/L	53.23	50.83	47.53	47.51	2.78	0.403	0.371	0.067	0.118	0.114	0.668	0.163
Propionate, mmol/L	12.98	12.23	11.80	11.87	0.72	0.644	0.258	0.517	0.261	0.259	0.515	0.240
Acetate:Propionate	4.06	4.16	4.08	4.06	0.09	0.873	0.262	0.069	0.329	0.852	0.569	0.742
Butyrate, mmol/L	7.10	7.05	6.80	6.73	0.42	0.897	0.264	0.355	0.329	0.471	0.971	0.625
Isobutyrate, mmol/L	0.84^{a}	0.80^{ab}	0.77^{bc}	0.73^{c}	0.02	0.009	0.887	0.962	0.865	0.001	0.800	0.009
Isovalerate, mmol/L	0.86^{a}	0.84^{ab}	0.78^{ab}	0.73^{b}	0.03	0.025	0.984	0.605	0.924	0.003	0.556	0.040
Valerate, mmol/L	1.10	1.11	1.06	1.07	0.05	0.898	0.395	0.728	0.226	0.555	0.960	0.712
BCVFA, mmol/L	9.90	9.80	9.41	9.25	0.47	0.742	0.324	0.470	0.351	0.292	0.942	0.472
NH3-N, mg/L	4.77	4.93	4.01	3.74	0.49	0.262	0.128	0.541	0.339	0.074	0.668	0.345
Protozoa, Log10/mL	5.06^{a}	4.98^{b}	4.98^{b}	5.06^{a}	0.03	0.045	0.185	0.035	0.887	0.926	0.005	0.094
BUN, mg/dL	23.00	21.79	21.11	19.65	1.32	0.334	0.485	0.327	0.156	0.075	0.926	0.160
Urinary N excretion[4], g/d	72.00	67.77	66.16	61.40	4.22	0.343	0.818	<0.001	0.081	0.080	0.951	0.155

[1]Dietary treatment values are given as least-squares means.
[2]If there were significant ($P \leq 0.05$) interactions between dietary treatment, and DMI the dietary treatment means are reported with the covariate structure in the model.
[3]Contrasts: L = Linear; Q = quadratic; QT = no Quebracho vs. Quebracho inclusion
[4]Estimated using the equation proposed by Kohn et al. (2005)

Table A-7. Effect of quebracho tannin extract upon in vitro gas production dynamic

	Dietary Treatment[1]					*P*-value		Constrasts[2]		
Items[3]	QT0	QT1	QT2	QT3	SEM	TRT	TRT x Day	Linear	Quadratic	C x QT
Exponential										
TGP, ml	18.14	17.41	18.08	16.64	0.55	0.058	0.013	0.051	0.405	0.127
kd, %/h	5.06	5.33	5.43	5.22	0.13	0.252	0.007	0.328	0.081	0.091
Lag time, h	-0.13	-0.03	-0.13	-0.09	0.18	0.404	0.953	0.262	0.310	0.101
Log. Two-Pool										
Asymptote (P1), ml	6.50[ab]	6.61[ab]	6.98[a]	5.89[b]	0.21	0.003	0.015	0.093	0.003	0.978
kd P1 (%/h)	9.98	10.59	10.64	10.72	1.03	0.380	0.398	0.138	0.421	0.088
Lag time to P2, h	1.76	2.33	2.09	2.18	0.19	0.186	0.854	0.227	0.200	0.047
Asymptote (P2), ml	10.87	10.25	10.56	10.04	0.37	0.260	0.339	0.121	0.861	0.107
kd P2, %/h	2.48	2.54	2.51	2.60	0.10	0.600	0.039	0.267	0.813	0.359
Exp. kd equivalent, %/h	4.17	4.27	4.19	4.42	0.20	0.532	0.043	0.265	0.629	0.421
Energy Estimates										
ivNDFD, 48h	45.02	46.25	49.40	52.75	2.11	0.063	0.441	0.009	0.618	0.077
CGP, ml/g NDFD	364.8[a]	321.0[ab]	304.4[ab]	256.0[b]	20.5	0.027	0.525	0.004	0.584	0.033
Methane, mg/g FOM	11.76	11.40	12.65	10.74	1.72	0.148	0.001	0.489	0.190	0.808
Methane, mg/g NDF digested	38.21	33.49	38.60	29.07	4.08	0.291	0.028	0.054	0.350	0.134
TDN, %	44.86	46.58	46.22	45.49	0.44	< 0.001	< 0.001	0.182	< 0.001	< 0.001
ME, Mcal/kg (TDN, 4%)	1.62	1.69	1.67	1.64	0.01	0.001	< 0.001	0.303	< 0.001	< 0.001

[a-b] Least Squares means in a row with different superscripts differ at $P \leq 0.05$.

[1]Dietary treatment values are given as LSM.

[2] C x QT = no Quebracho vs. Quebracho inclusion

[3]TGP = Total gas production of the exponential nonlinear function; kd = the fractional rate of gas production of the exponential nonlinear function; Lag time = time required to commence fermentation; Asymptote (P1) = accumulative gas production of nonfiber carbohydrate pool (NFC); kd (P1) = fractional rate of gas production of NFC pool; Lag time to P2 = time required to commence fermentation of fiber carbohydrate (FC) pool; Asymptote (P2) = accumulative gas production of FC pool; kd P2 = fractional rate of gas production of FC pool; Exp. kd equivalent = exponential decay digestion rate (kd); ivNDFD = in vitro neutral detergent fiber digestibility; CGP = cumulative gas production, ml per gram of NDF digested; TDN = computed TDN; ME = metabolizable energy

APPENDIX B

FIGURES

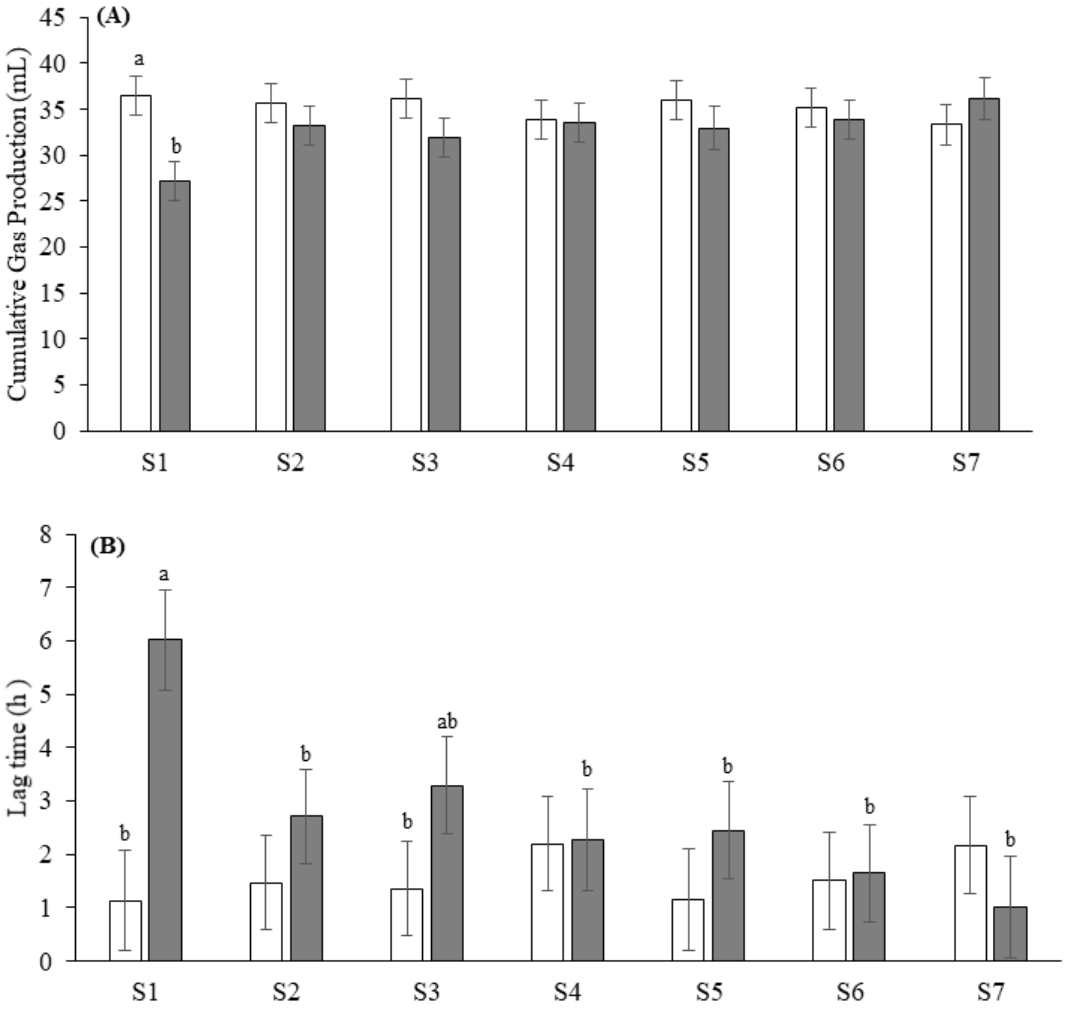

Figure B-1. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on in vitro (A) cumulative gas production and (B) lag time of the nonlinear exponential function [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

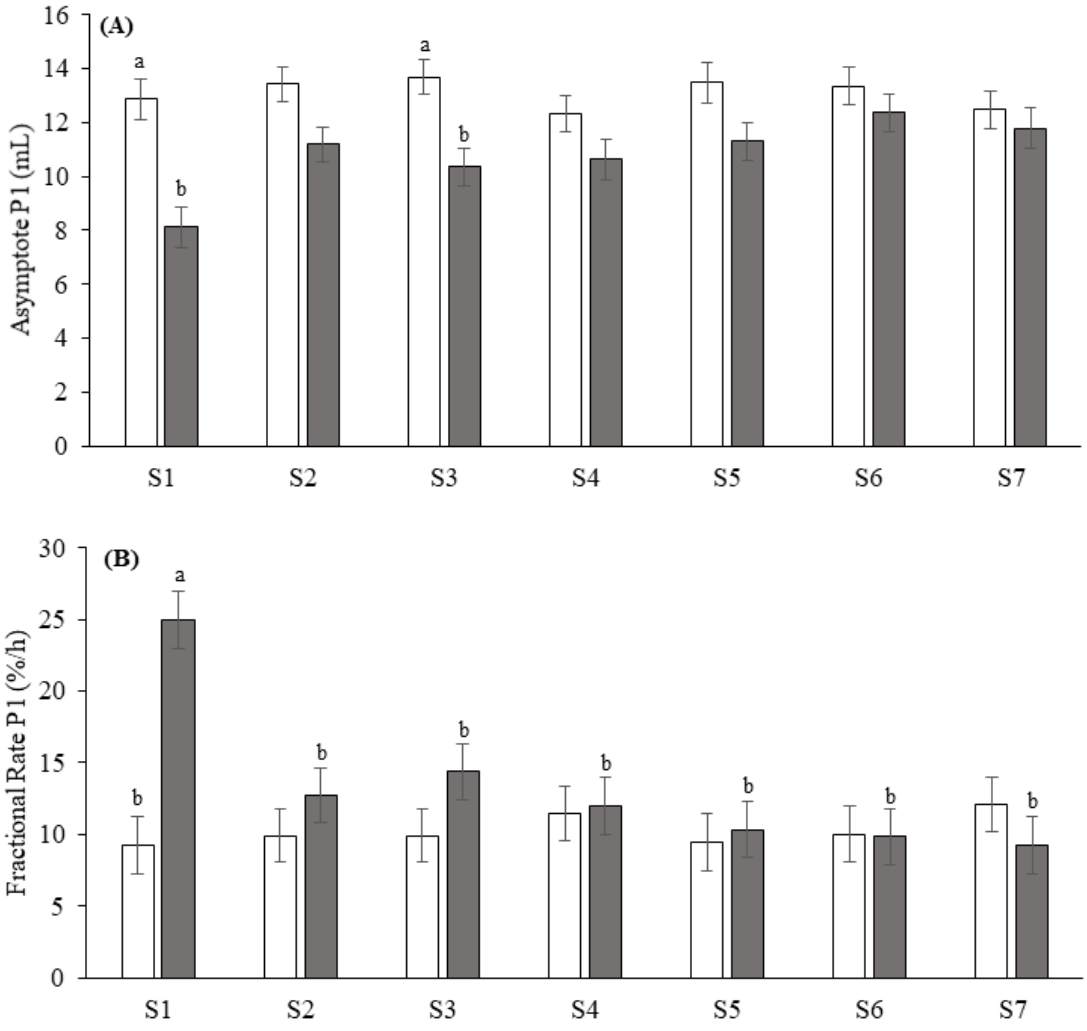

Figure B-2. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on in vitro (A) asymptote P1 = accumulative gas production of nonfiber carbohydrate pool (NFC) and (B) fractional rate P1 = fractional rate of gas production of NFC pool [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

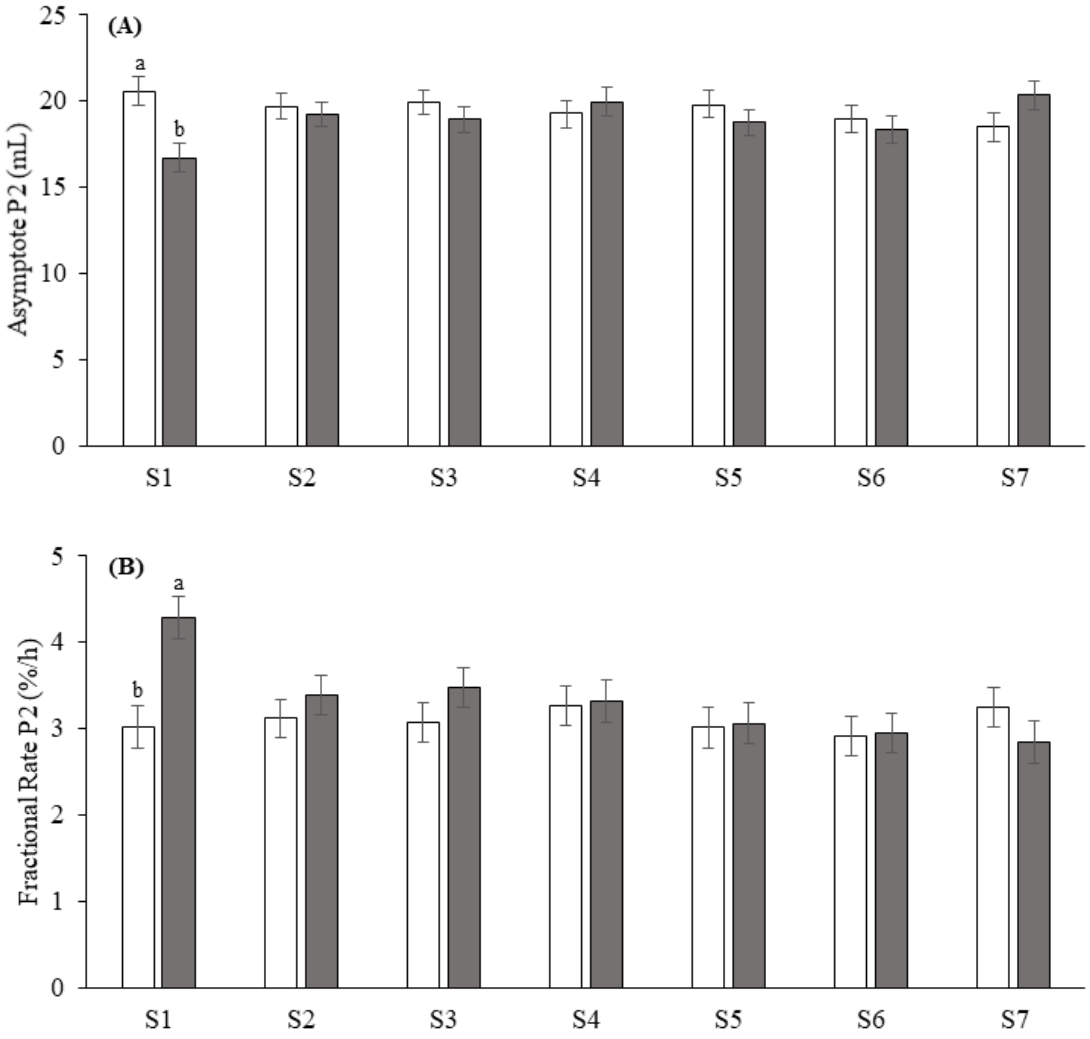

Figure B-3. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on in vitro (A) asymptote P2 = accumulative gas production of fiber carbohydrate (FC) pool and (B) fractional rate P2 = fractional rate of gas production of FC pool [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

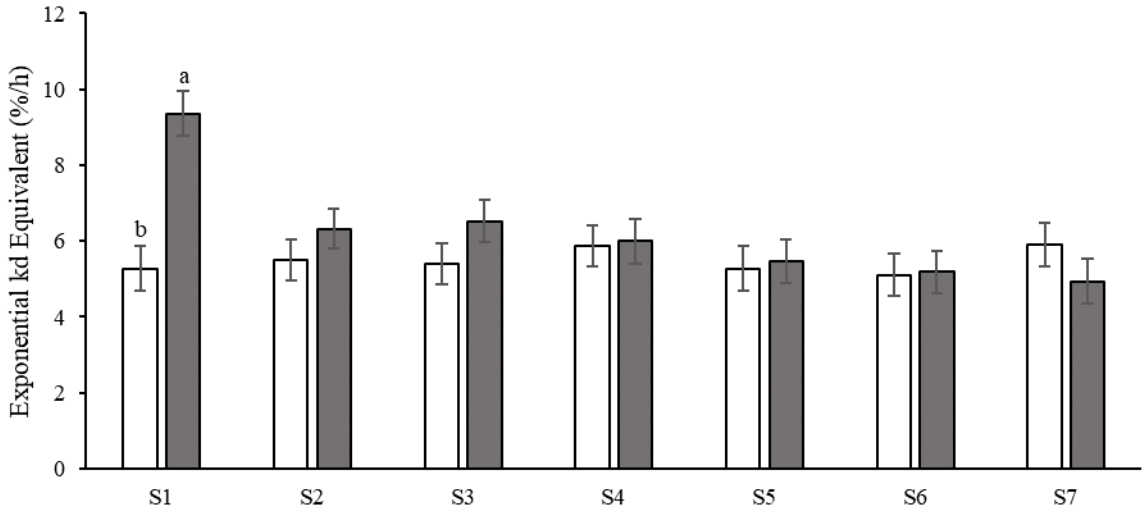

Figure B-4. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on exponential decay digestion rate (kd) [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

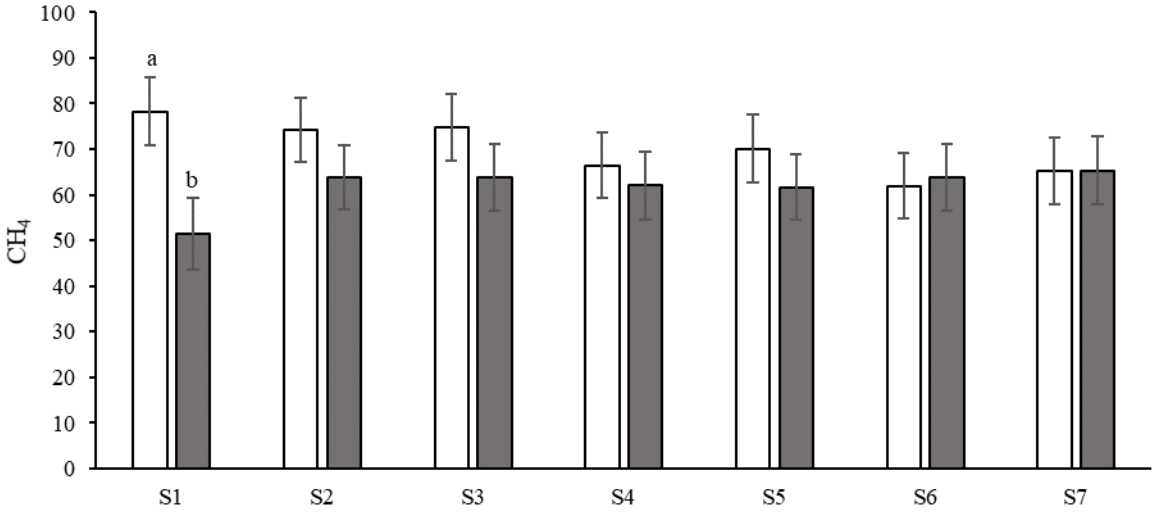

Figure B-5. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on CH_4 = methane production (mg/g FOM) [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

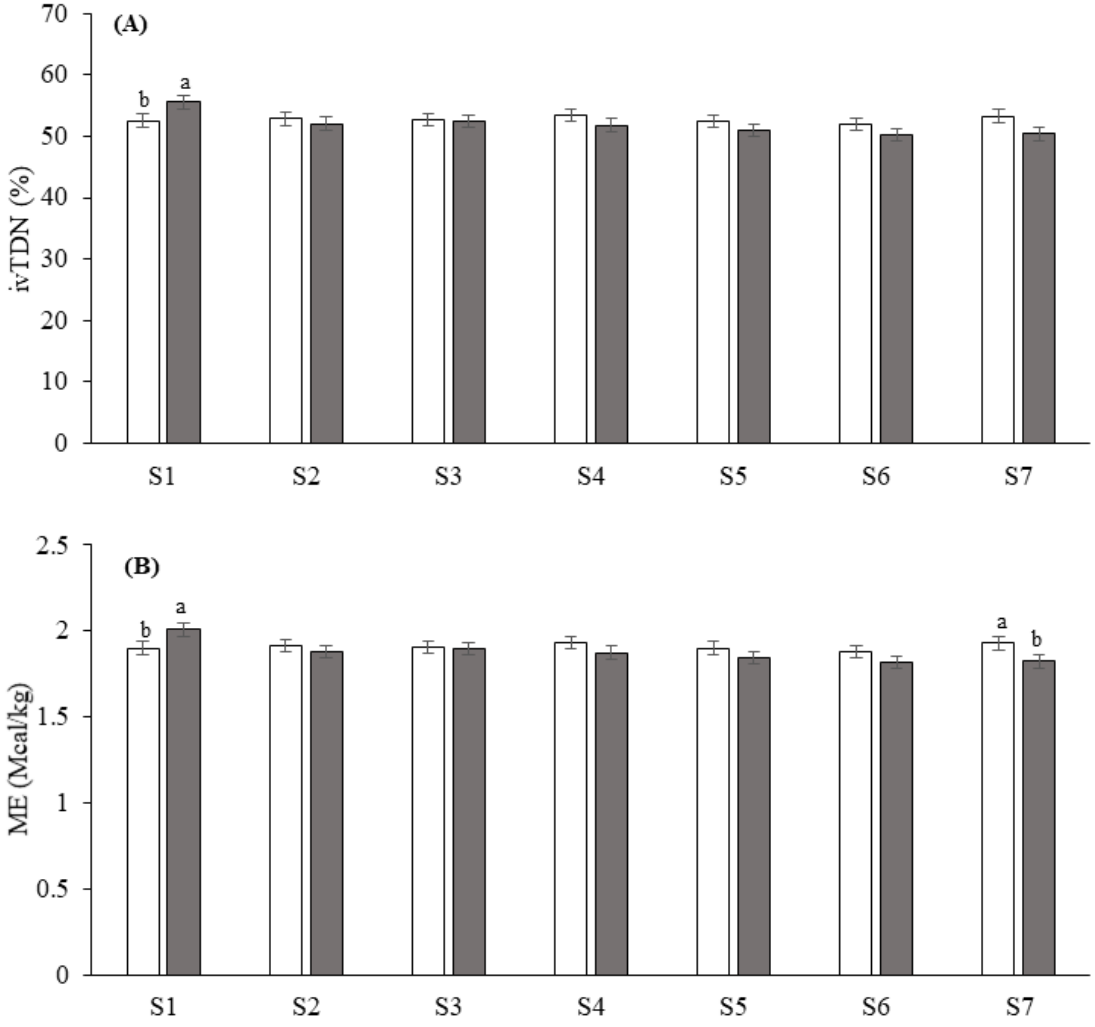

Figure B-6. Effects of seven different encapsulation methods of branched-chain volatile fatty acids (BCVFA) salts incubated without the addition of casein and cysteine on in vitro (A) TDN = computed total digestible nutrient and (B) ME = metabolizable energy [white = L3 (3% of inclusion, DM basis), gray = L6 (6% of inclusion, DM basis)].

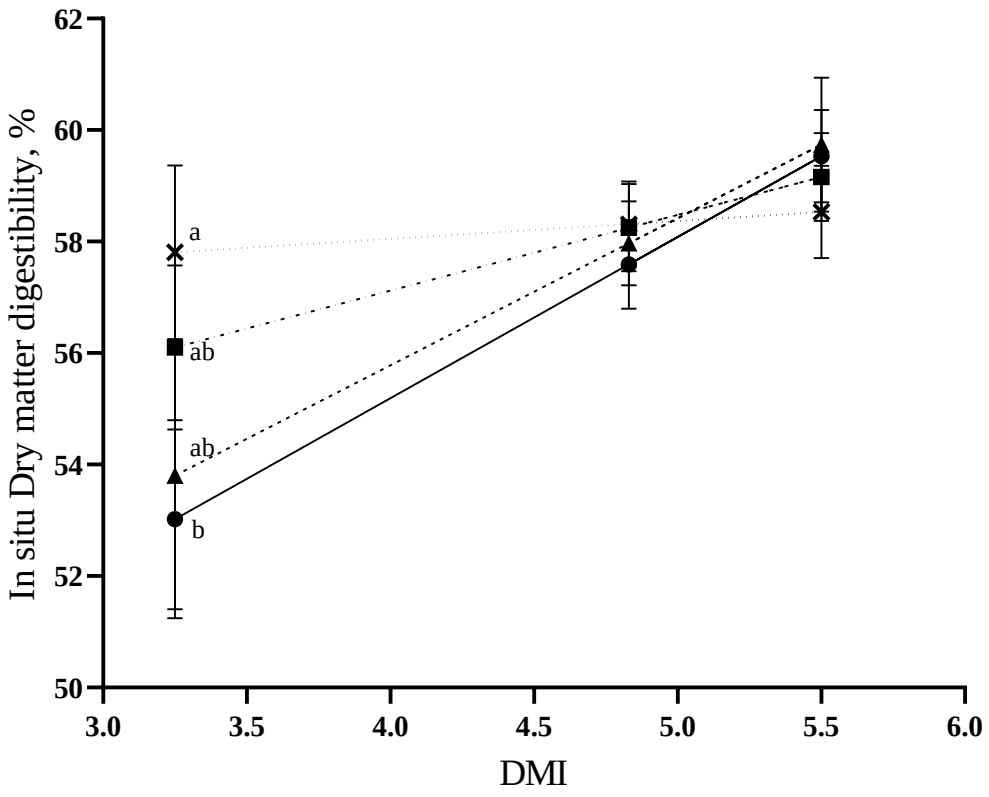

Figure B-7. Effect of quebracho tannin extract (QT) upon in situ dry matter digestibility on different intake levels (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).

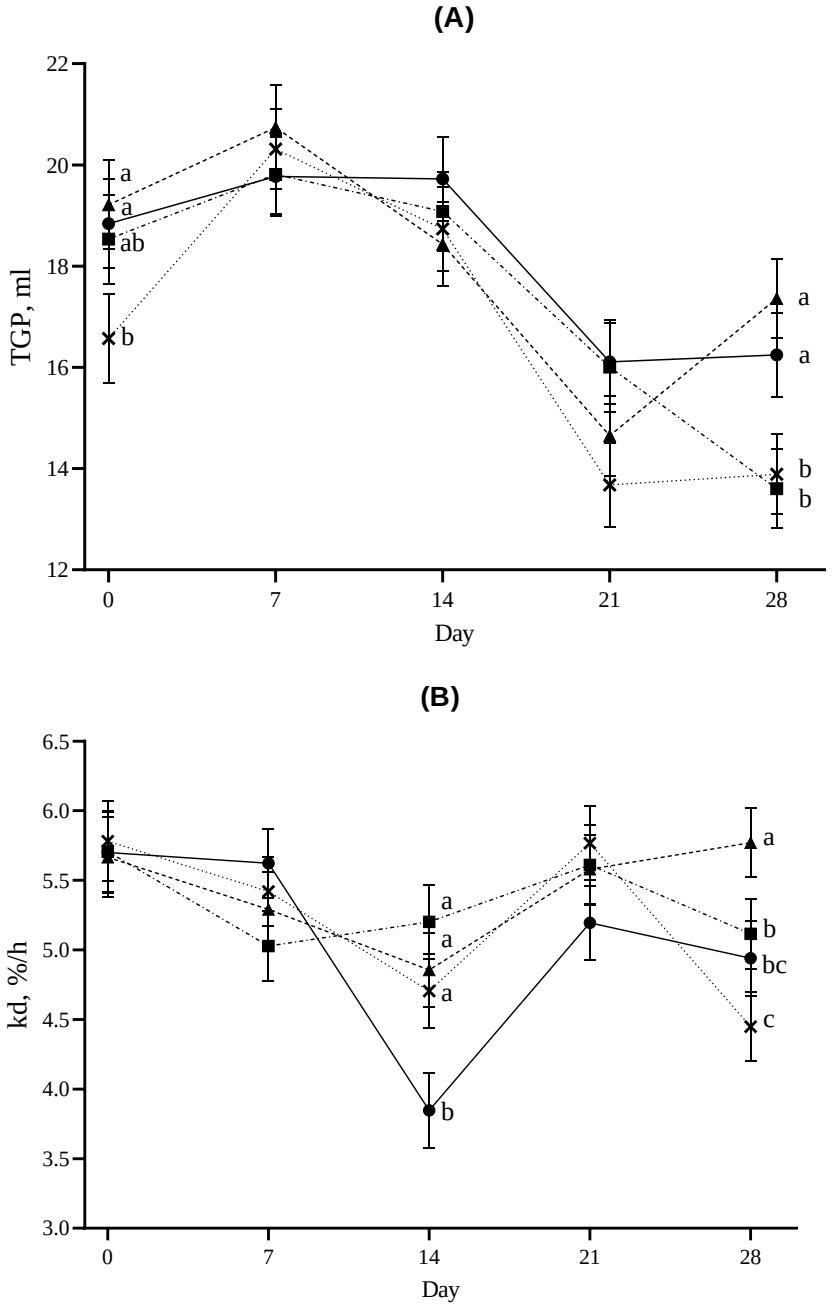

Figure B-8. Effect of quebracho tannin extract (QT) inclusion on (A) Total gas production of the exponential nonlinear function and (B) the fractional rate of gas production of the exponential nonlinear function (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).

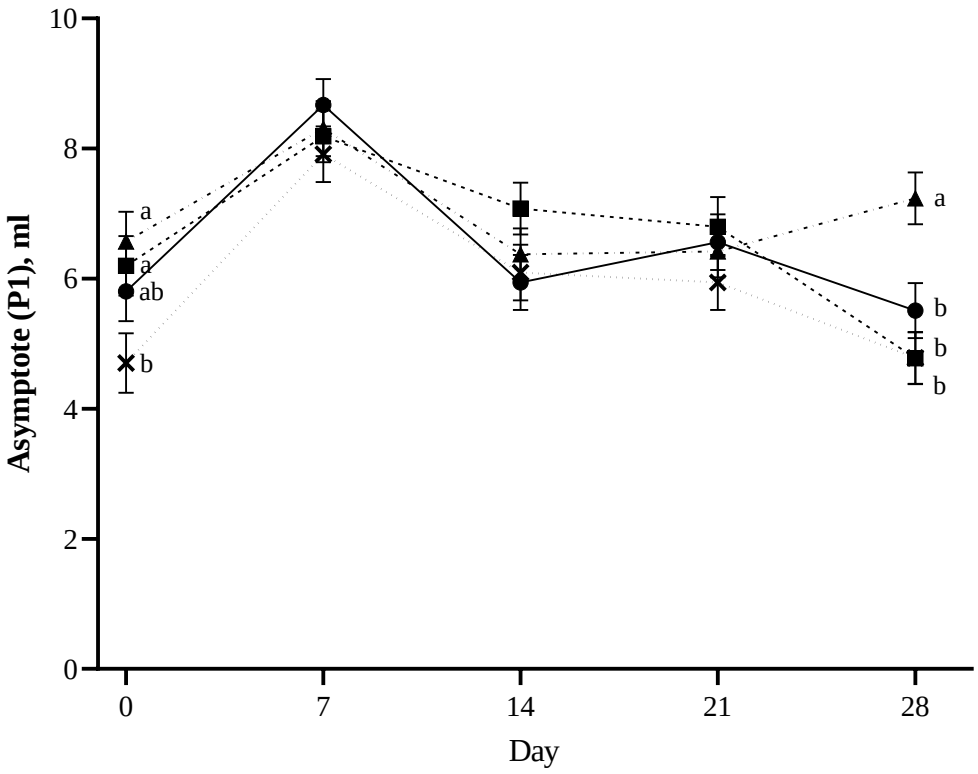

Figure B-9. Effect of quebracho tannin extract (QT) inclusion on accumulative gas production of nonfiber carbohydrate pool (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).

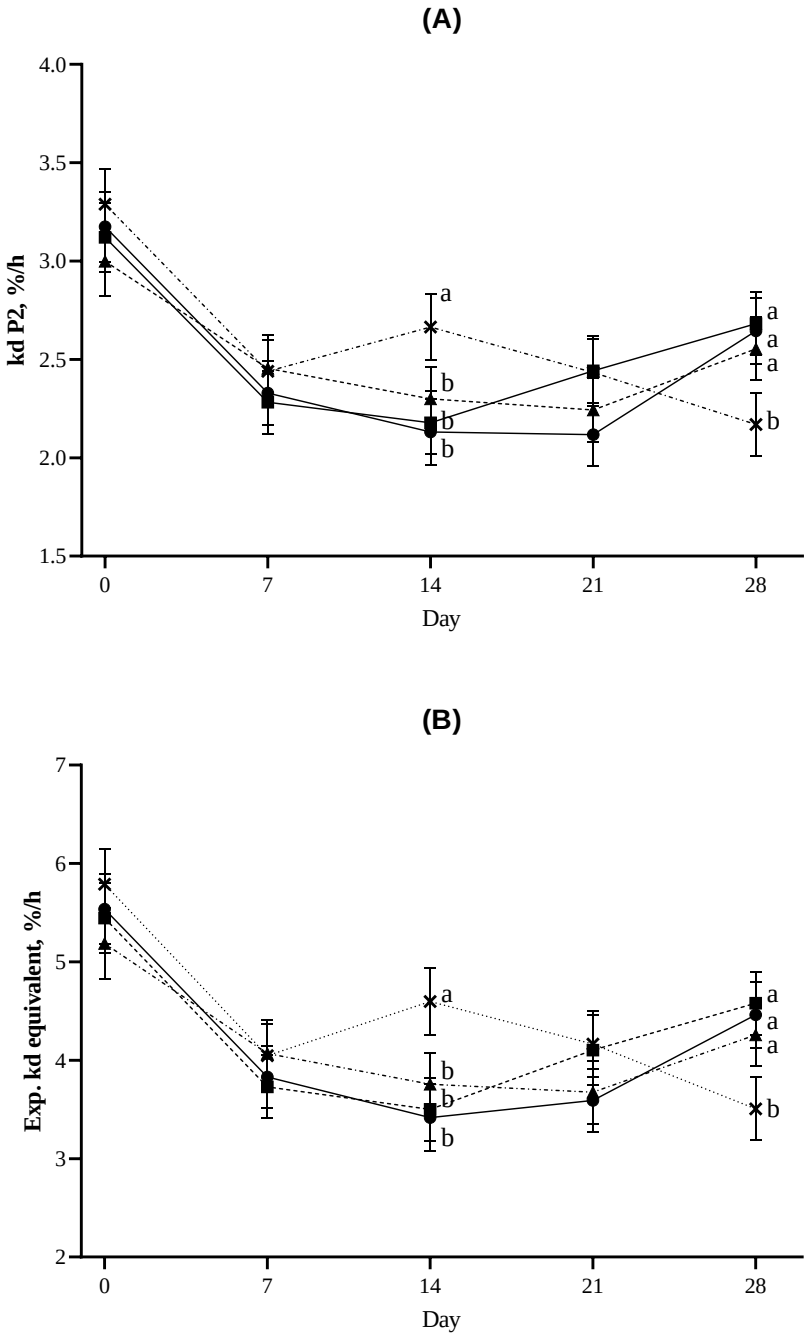

Figure B-10. Effect of quebracho tannin extract (QT) inclusion on (A) fractional rate of gas production of FC pool and (B) the exponential decay digestion rate (kd) (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).

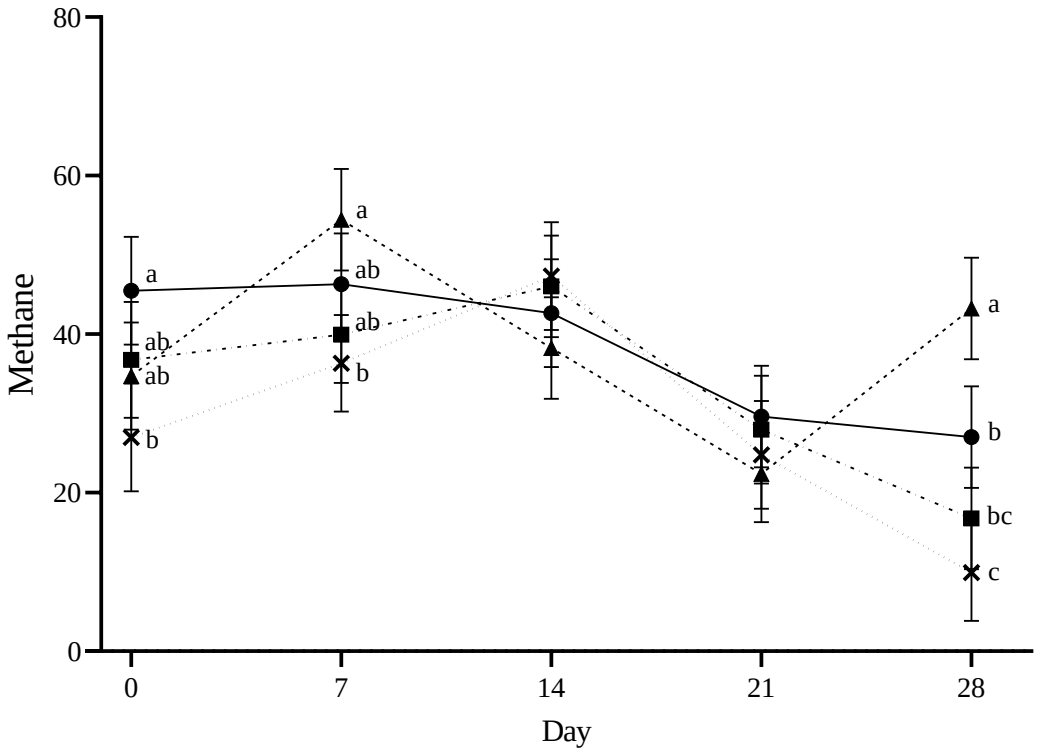

Figure B-11. Effect of quebracho tannin extract (QT) inclusion on methane production (mg/g NDF Digested) (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).

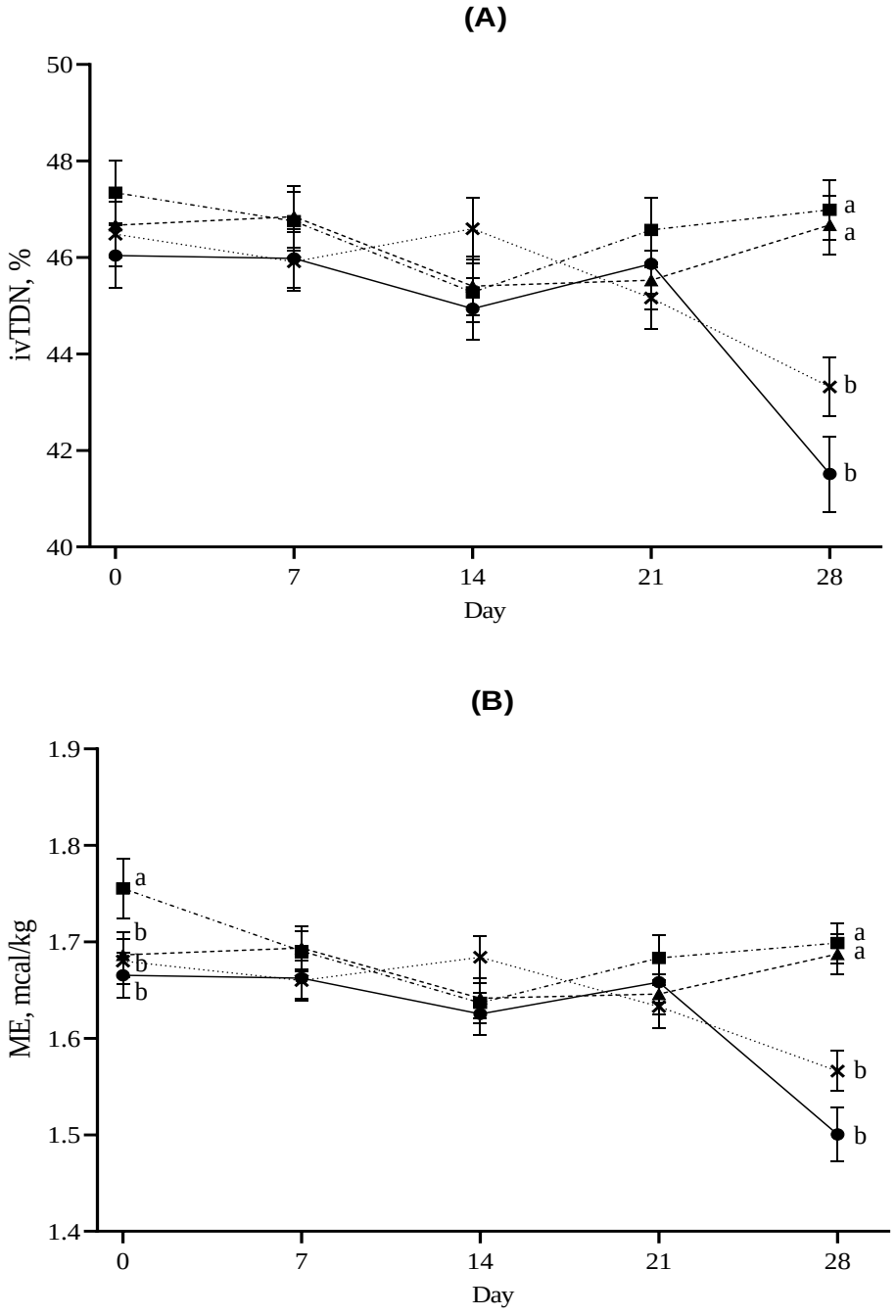

Figure B-12. Effect of quebracho tannin extract (QT) inclusion on (A) computed total digestible nutrient and (B) metabolizable energy computed (•, solid line = QT0 (0% DM), ■, long dash dot = QT1 (1% DM), ▲, long dash = QT2 (2% DM), ×, round dots = QT3 (3% DM)).