12-2021

Effects of Cashew Nut Shell Extract on Nutrient Digestibility and Ruminal Fermentation Under in Vitro Batch Culture and Continuous Culture Conditions

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EFFECTS OF CASHEW NUT SHELL EXTRACT ON NUTRIENT DIGESTIBILITY AND RUMINAL FERMENTATION UNDER IN VITRO BATCH CULTURE AND CONTINUOUS CULTURE CONDITIONS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Sciences

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by
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December 2021

Accepted by:
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ABSTRACT

The overall objective of this research was to determine the effects of cashew nut shell extract (**CNSE**; 59% anacardic acid and 18% cardol) on nutrient digestibility and rumen fermentation profile under in vitro batch culture (Chapter 2) and continuous culture conditions (Chapter 3). The second objective was to determine an optimal supplementation dosage for dairy cows. The batch culture study was organized as a randomized complete block design with 15 replicates per treatment incubated for 24 h during four incubation runs. Each incubation was inoculated with rumen contents collected from ruminally fistulated cows fed either a close-up (**CU**; 15.1% CP, 38.1% NDF, and 20.8% starch) or a fresh cow diet (**FC**; 16.4% CP, 31.8% NDF, and 28.0% starch). Diets fed to donor cows were the same as the substrate used in the batch culture incubations. Treatments consisted of four levels of granulated CNSE formulated to contain 50% CNSE (additional 50% representing the coating and carrier) and added to the cultures in increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d. Dietary treatments incubated to mimic CU conditions had no impact on in vitro dry matter digestibility (**IVDMD**), in vitro true dry matter digestibility (**IVTDMD**), in vitro neutral detergent fiber disappearance (**IVNDFD**), and total nitrogen digestibility (**TNd**). Under CU conditions, increasing CNSE level decreased the molar proportion of acetate (linear and quadratic effect), propionate (linear effect), increased isovalerate and valerate, and pH (linear and quadratic effect). When mimicking FC conditions, increasing the level of CNSE tended to linearly decrease IVDMD, IVTDMD, IVNDFD, and TNd. Acetate molar proportions tended to decrease (linear effect), whereas isovalerate and valerate

showed dose-dependent increase (linear effect). Ammonia nitrogen (**NH_3N**) decreased at all levels of supplemented CNSE in a linear and quadratic manner. Culture pH increased at 2.5 and 5.0 g of CNSE/head/d and decreased at 10 g of CNSE/head/d (quadratic effect). Under batch culture conditions, adding incremental levels of CNSE had no effect on nutrient digestibility in CU diets, but negatively impacted digestibility in FC diets.

Under continuous culture conditions, four treatments were randomly assigned to eight fermenters for two periods of 10 d. Treatments consisted of four doses of granulated CNSE formulated to contain 50% CNSE, premixed with corn grain, and added to diets in stepwise increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d. Fermenters were fed 56.2 g/d of a total mixed ration (**TMR**; 17.0% crude protein (**CP**), 29.7% NDF, and 29.9% starch), divided between two feedings at 0800 and 2000 h. The apparent digestibility of dry matter (**DM**), organic matter (**OM**), and neutral detergent fiber (**NDF**) were not affected by CNSE supplementation. Cashew nut shell extract had no effect on total volatile fatty acids (**VFAs**) or individual VFA molar proportions, culture pH, oxidation-reduction potential (**E*h***), or relative hydrogen score (**rH)**. These results suggest that CNSE has no impact on nutrient digestibility and rumen fermentation profile under continuous culture conditions.

DEDICATION

I dedicate this thesis to my husband, Timothy Wendel, my parents, Brian and Edna Bowen, my siblings, Harrison Compton and Logan and Coker Bowen, and my grandmother, Edna Coker, for their endless advice, unconditional patience, sacrificial love, and for pointing me towards Jesus in all circumstances. You all have helped me achieve this milestone in my life.

ACKNOWLEDGMENTS

I want to express my gratitude to my committee chair, Dr. Matias Aguerre. Having Dr. Aguerre as my mentor has been the biggest blessing, and I will forever be grateful for his enthusiasm, leadership, and optimism. I was once told, "A good scientist can help you create better experiments, a big name can give you connections, and a good writer will help you write better papers, but a PI who is kind will make you a better person." Dr. Aguerre possesses all of these characteristics; however, I am most thankful for his kindness. I don't know if I could have made it through graduate school without the kindness, patience, and support you've shown me. It has been an honor to work for you.

I want to acknowledge my committee members, Dr. Gustavo Lascano and Dr. James Strickland, for their support and insights. Dr. Lascano, thank you for challenging me and taking me out of my comfort zone. Through your expertise, you have helped me to grow as a scholar. Dr. Strickland, you have been essential for my growth here at Clemson. I will forever be grateful for you believing and investing in me since my undergraduate career. I have enjoyed being your teaching assistant the most. You have taught me how to interact and show compassion to undergraduate and graduate students and the ends and outs of taking on a leadership position. You have made me more confident because you have bestowed so much knowledge upon me. I am beyond thankful for you.

I would also like to thank the entire AVS department for their support over the six years I've been at Clemson. In particular, I would have been lost without Stacy Miller and Lauren St Clair. Thank you for sharing the role of "School Mom" for advising me

about the department and life in general. You have never hesitated to lend a hand; I am thankful for everything you have done to help me succeed.

I have been fortunate to spend most, let's be honest, all of my life thus far in Clemson, SC. Graduate school introduced me to a wonderful group of people who have enriched my life and made my extended time here unforgettable. Special thanks to Manuel. You have been my best friend here since day one. Our friendship was instant. You have brought so much joy ("why do we always say stupid stuff"), laughter (name a time when we weren't laughing), and culture (the Colombian King) in my life. Thank you for all the chats, scientific discussions, and all the memories I will cherish forever. You are the hardest worker I know, and I will be forever thankful for your help on my projects. You're a friend who has become family. Speaking of family, I can't forget my academic sister Monica. You are the best officemate ever and such a great friend! During all the stressful moments, you were there. Thank you for helping me grow academically. I am going to miss all of the laughs that we shared. Dana, I remember the first day I met you in journal club. Ever since that day, you have been a dear friend to me. Thank you for being someone I can always depend on in my personal life and throughout my academic journey.

To my family, my academic endeavors would not have been possible without all of you. My husband, Tim, thank you for your presence throughout this entire process; this adventure would not have been thinkable without you by my side. You have supported me every step of the way – even when it has come to personal sacrifice. I am so thankful that you are my forever teammate; I look forward to our future together and

the adventures it entails. Brain and Mom, you have always been there to keep me grounded. You both supported my pursuit of Animal and Veterinary Science during my undergraduate studies and were supportive throughout my graduate career as well. I owe a great deal of who I am to the two of you. To my siblings, Harrison, Logan, and Coker, my life would be incomplete without all of you; I have a different but very special relationship with each one of you. You three have always kept me laughing and are indeed my best friends. Finally, to my grandmother, Edna Coker, you have been the foundation of our family. I am so blessed that I had the opportunity to pursue higher education at Clemson, as it allowed me to spend a great deal of time with you. I have enjoyed our weekly lunch dates and will forever cherish the memories we have created together. No matter where life takes me, I will always be there for you, just as you have been for me all of these years. I'll love you forever.

Thanks again to everyone who made this thesis possible.

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CHAPTER ONE

REVIEW OF LITERATURE

Introduction

Humanity's greatest challenge is feeding a global population estimated to reach nine to eleven billion over the next 30 years (Fróna et al., 2019). Doing that, and alleviating the hunger that is present today, will require a 70 percent increase in global agricultural production by 2050 (Fróna et al., 2019). To meet this challenge, food producers will need to increase productivity while preserving natural recourses (land, water, air) that make agricultural production possible. Ruminants play an important role in human food production because they convert feedstuffs unsuitable for humans (e.g., waste byproducts, high fiber forages) into food products such as meat and milk (Hofmann, 1989). Nutrient utilization determines how efficiently ruminants can convert feedstuffs into human edibles. As a result of efficient nutrient utilization, less feed is required to meet the animal's production needs. Fewer waste nutrients are released into the environment, potentially improving fresh and marine water quality. The producer saves money by improving nutrient utilization, which can then be used in other production areas.

Because feed ingested by ruminants is first available for fermentation by the rumen microorganisms, research on influencing ruminal microbial population has been studied extensively (Watanabe et al., 2010; Patra and Saxena, 2011; Kobayashi et al., 2016). All productive processes are impacted by rumen fermentation; therefore, maximizing rumen efficiency is critical and directly impacts productivity. Ruminants

have an interdependent relationship with their microorganisms; for example, the ruminant provides dietary nutrients and an environment for the digestion of feedstuffs, and the microorganisms increase the productivity of cellulolytic feeds (Van Soest, 1982a). However, there are inefficiencies in the rumen because microbial fermentation yields energy and protein losses (mainly methane and ammonia N, respectively). These inefficiencies release waste products into the environment and reduce production performance (Tamminga, 1996). Manipulation of ruminants' diets to change the types of metabolic products obtained from the metabolism of carbohydrates, proteins, and other nutrients is one approach for improving rumen microbial efficiency.

Nutritional strategies, such as supplementing with feed additives, provide a competitive advantage for specific microbial populations, thus improving feed efficiency and production performance. Traditionally, ionophore antibiotics (e.g., monensin, lasalocid, and laidlomycin propionate) have been fed to ruminants because of their ability to successfully alter rumen fermentation (Russell and Strobel, 1989). However, due to public concerns surrounding ionophores usage, researchers are looking for alternatives to improve rumen efficiency (McGuffey et al., 2001). As a result, many countries have placed restrictions on the use of antibiotics in livestock, and the European Union (Regulation 1831/2003/EC) has even outlawed them. Therefore, ruminant nutritionists are becoming increasingly interested in using natural alternatives such as tannins, essential oils, cashew nut shell extract, and other organic compounds as a way to replace antibiotics to lessen worries about human health risks.

For this thesis's purpose, cashew nut shell extract (CNSE) was evaluated in batch culture and continuous culture experiments to determine if CNSE could be a useful feed additive for improving nutrient digestibility and rumen fermentation. It's worth noting that the terms cashew nut shell liquid (CNSL) and cashew nut shell extract (CNSE) are used interchangeably in the literature and refer to the product's mechanical cold-pressed extraction procedure. The term CNSE will be used throughout this thesis to avoid confusion.

This chapter will first give a general overview of rumen fermentation and methodologies to measure rumen fermentation in vitro, emphasizing batch, semicontinuous, and continuous cultures. Lastly, a brief overview is presented on ionophores and alternative additives such as secondary metabolites, more extensively CNSE, and its impact on nutrient digestibility and rumen fermentation.

Overview: Rumen Fermentation

Grazing ruminants (i.e., grass and roughage eaters) have a four-chambered stomach capable of digesting fibrous plant material (Hofmann, 1989). The first three anatomical chambers (rumen, reticulum, and omasum) are commonly referred to as the forestomachs (Van Soest, 1982a). Microorganisms colonize the forestomachs and the large intestine; therefore, fermentation (i.e., digestion by microbial action) can occur. Gastric digestion occurs in the fourth compartment (i.e., abomasum) and small intestine. The abomasum produces hydrochloric acid and digestive enzymes (e.g., pepsin) that aid in nutrient breakdown and nutrient absorption. Dairy cattle are categorized as pre-gastric fermenters because the microbial populations ferment feedstuffs before the feed is

exposed to the enzymes secreted by the abomasum and the small intestine (Hofmann, 1989).

The majority of fermentation occurs in the largest compartment (i.e., rumen). The rumen is an anaerobic fermentation chamber that provides the microorganisms with: (1) nutrients, (2) constant temperatures, (3) maintain pH, and (4) a buffered environment that enhances microbial activity and growth (Van Soest, 1982a). The rumen ecosystem involves thousands of species of bacteria (10^{10}-10^{11} cells/mL), archaea (10^{7}-10^{9} cells/mL), protozoa (10^{4}-10^{6} cells/mL), fungi (10^{3}-10^{6}), and viruses (10^{9}-10^{10}) (Wright and Klieve, 2011). The proportion in which these respective populations co-exist depends primarily on diet (e.g., forage vs. concentrate) and turnover rate (Van Soest, 1982b). Bacteria are primarily anaerobic and are classified into three functional groups: amylolytic, cellulolytic and proteolytic types (Van Soest, 1982b). Amylolytic bacteria ferment nonstructural carbohydrates (e.g., organic acids and starches), cellulolytic bacteria ferment structural carbohydrates (e.g., hemicellulose and cellulose), and proteolytic bacteria ferment proteins. Amylolytic bacteria thrive at a pH of 5.8 to 6.5, whereas cellulolytic bacteria flourish at a pH of 6.7 (Van Soest, 1982b). Nonstructural carbohydrates are highly fermentable; however, ruminants cannot digest structural carbohydrates because they cannot produce respective hydrolytic enzymes (Krause et al., 2003). Therefore, the host depends on the microorganisms to hydrolyze these compounds (e.g., break the beta 1-4 bonds between glucose monomers) to generate energy for themselves and the animal (Bergman, 1990).

Microbial fermentation alters the nutrient profiles of the initial feed; in other words, nutrients absorbed differ from the nutrients consumed. Carbohydrates are the most abundant substrate for rumen bacteria. Microbes ferment plant fibers (cellulose, hemicellulose) and starch consumed into volatile fatty acids (VFAs): mostly acetate, propionate, and butyrate, which supply most of the energy to the ruminant once absorbed across the rumen wall (Bergman, 1990). However, carbohydrate degradation results in energy losses (e.g., hydrogen (H_2) and carbon dioxide (CO_2)) and these byproducts can be utilized as sources of energy by methanogens to synthesize methane (CH_4) (Krause et al., 2003). When protein is exposed to the microorganisms in the rumen, it is quickly broken down to ammonia (NH_3). The microbes use NH_3, coupled with energy generated from carbohydrate metabolism to synthesize microbial protein (MP) (Maeng and Baldwin, 1976). However, carbohydrates and protein are also converted into VFAs, NH_3, CH_4, and CO_2. As a result, synchronizing energy release and protein degradation may increase the yield of MP synthesis and growth (Sinclair et al., 1993). Non-protein nitrogen (NPN) sources, such as urea, can also be converted into NH_3, which the microbes can use to form MP as well. A portion remains undegraded (rumen undegradable protein) by the microorganisms in the rumen and gets broken down into amino acids (AA) later in the digestive tract. Endogenous protein arises from saliva, digestive enzymes, and epithelial cells and is another source of protein for the animal, but digestion can be difficult to quantify (van der Walt and Meyer, 1987). Microbial fermentation creates end-products such as VFAs and NH_3, which supplies the majority of

the metabolizable energy and protein to the ruminant animal, respectively (Krause et al., 2003).

In Vitro Rumen Fermentation Techniques

Rumen fermentation is often estimated by measuring fermentation variables using in vitro procedures. In vitro systems are used to simulate the fermentation process in the rumen without the host. Many external factors influence the microorganisms and enzymes that reside in the rumen; therefore, in vitro techniques allow ruminant nutritionists to study a response when only one variable is changed. Thus, in vitro systems have been beneficial for evaluating feedstuffs, microbial fermentation, and feed additives (McIntosh et al., 2003). In vitro systems are quicker, offer better replication, and evaluate digestion processes with various complexity levels (Tilley and Terry, 1963; Teather and Sauer, 1988; Brandao and Faciola, 2019). However, oftentimes results of in vitro studies do not translate to commercial settings because the product being examined is too expensive to execute or the efficiency of the product cannot be proven under in vivo conditions (Yáñez-Ruiz et al., 2016).

The basis for all in vitro systems is anaerobic fermentation of a sample substrate, a medium (e.g., buffer solution simulating ruminant saliva), and rumen fluid pooled from donor animals followed by endpoint measurements (Mould et al., 2005). For in vitro models to be successful, it is essential to satisfy both environmental and nutritional requirements for the rumen microorganisms. Batch, semicontinuous, and continuous cultures are among the various in vitro models that exist.

Batch Cultures

Batch culture systems are the simplest way to evaluate the digestibility and fermentation of feedstuffs or additives in vitro. Batch in vitro uses substrates that are typically ground to 1mm, a medium that provides nutrients, and rumen fluid pooled from multiple donor animals. The donor animals are fed the same or a similar diet to the one fed in vitro to ensure microbial adaptation to the diet prior to inoculation. Tilley and Terry (1963) developed the batch culture technique using mixed cultures to determine forage digestibility and digestion rates. In addition, the system was designed to handle many samples or experimental treatments with minimal substrate. Briefly, the procedure consists of two-steps, incubating 0.5 g of substrate, 10 mL of rumen fluid, and 40 mL of a buffer solution for 48 h (Tilley and Terry, 1963). Afterward, the fermentation vessel was centrifuged, the supernatant was removed, and the residue was incubated in a pepsin solution (2 pH) for an additional 48 h. The remaining residue contained undigested plant cell wall plus bacterial debris, and apparent in vitro dry matter digestibility (IVDMD) was calculated from this procedure. Goering and Van Soest (1970) improved this procedure by exposing the fermented substrate to a neutral detergent solution instead of pepsin, ultimately removing all indigestible microbial matter. This modification made it possible to calculate the in vitro true dry matter digestibility (IVTDMD) in batch cultures (Goering and Van Soest, 1970).

Additionally, Czerkawski and Breckenridge (1975) created a method to measure fermentation gasses through a piston's displacement, and these findings created the framework for the 'Hohenheim gas test' (Menke et al., 1979). Menke et al.’s method was

later revised by incubating syringes in a water bath, which allowed gas output to be sampled more frequently (Blümmel and Ørskov, 1993). This modification allowed fermentation kinetics to be calculated in batch culture systems. Wilkins (1974) and Theodorou et al. (1994) measured gas accumulation using sealed vessels containing a substrate, buffer, rumen fluid, and a pressure transducer. Traditionally, substrate was added to the fermentation vessels; however, in 1992, ANKOM developed the Filter Bag Technology (FBT). Instead of the substrate being added directly to the vessel, the substrate is confined inside filter bags. This development makes fiber analysis easier and creates a more accurate way to measure variables under batch culture conditions. Rumen digestibility and fermentation can be evaluated using these batch in vitro techniques or a slight modification of these procedures.

Although batch cultures have been utilized for many years, this model has significant limitations compared to other in vitro techniques. While batch cultures provide an accurate way to measure substrate digestibility, the substrate available for fermentation becomes limiting over time. Batch cultures only ferment substrates for a short time, often 18 (Danielsson et al., 2014), 24 (Benchaar et al., 2007a) or 48 h (Brooks et al., 2014). These systems are not usually operated for longer than 48 h because the batch culture system cannot remove fermentation end products. In the ruminant, for example, following carbohydrate fermentation, VFAs are primarily absorbed across the rumen epithelium, but without proper removal, the buffer capacity diminishes as VFAs accumulate. Despite the limitations of in vitro batch cultures, these systems are valuable for testing preliminary hypotheses.

Semicontinuous Cultures

Semicontinuous cultures are another in vitro approach for evaluating ruminal fermentation patterns, experimental treatments, and feed additives in vitro. The rumen simulation technique (RUSITEC) is the most widely utilized semicontinuous culture system in ruminant research (Mateos et al., 2017). The RUSITEC model was created by Czerkawski and Breckenridge (1977) to analyze ruminal fermentation while decreasing environmental variability over longer periods of time. Unlike batch cultures systems, the RUSITEC model can be kept running for several days and it allows for more precise control over environmental factors (e.g., pH, temperature, and buffer flow) (Mateos et al., 2017; Wetzels et al., 2018). Semicontinuous systems are cheaper, offer better replication, require a smaller number of animals and yield quicker results compared to in vivo.

Although the RUSITEC is a standardized in vitro approach, it has drawbacks when compared to continuous cultures and in vivo methods. In contrast to continuous cultures, the RUSITEC retains the feed substrate in a nylon bag, and the bag gets replaced on subsequent days, limiting its application to estimations of apparent digestibility of nutrients (Czerkawski and Breckenridge, 1977; Hristov et al., 2012). Other limitations include differences in the flows for liquids and solids, reduced VFA concentrations and protozoal shifts, and the lack of absorption capacity for metabolites compared to in vivo (Wetzels et al., 2018).

Continuous Cultures

Continuous culture systems have been used since the late 1950s and early 1960s to simulate rumen conditions in vitro. However, these systems have been modified to

address several limitations (Slyter et al., 1964; Teather and Sauer, 1988). Continuous cultures are used to evaluate nutrient digestibility, microbial fermentation and gas production in vitro. Continuous culture fermenters are reaction vessels capable of longer fermentation, with incubation periods varying from 7 d (Watanabe et al., 2010) to 11 d (Dai et al., 2019). The cultures are kept anaerobic by the continuous addition of CO_2 to displace O_2 that may enter during feeding and sample collection. The system can maintain a sufficient pH range (6.0-6.5) for microbial growth and reproduction by continuously adding buffer to the cultures. A recirculating water bath prevents the inoculum temperature from fluctuating and keeps internal temperatures around 39ºC to maximize fermentation. Compared to other in vitro systems, continuous cultures can remove fermentation end products through an overflow port and collection flask. This prevents the accumulation of fermentation products (e.g., VFA and NH_3) remaining in the culture and inhibiting fermentation.

Compared to animal trials, continuous cultures cannot remove excess acid produced from microbial fermentation (Van Soest, 1982a). In the rumen, most excess acid is absorbed across the rumen epithelium (Van Soest, 1982a); however, the glass vessel makes this biological process impossible. Therefore, removal is entirely dependent on washout via the overflow port (Van Soest, 1982a). Lack of absorption capacity leads to the buildup of fermentation end products and a decline in microbial population (Van Soest, 1982a). Warner (1956) maintained a microbial population using a dialysis sac for 4 d *in vitro*; but the microbial populations decreased because the fermentation products accumulated inside the sac. Additionally, Hristov et al. (2012) observed lower OM and

NDF digestibility, lower total and individual VFA concentrations in continuous cultures compared to in vivo studies. Likewise, Mansfield et al. (1995) reported higher concentrations of cellulolytic bacteria and protozoa in vivo. Contrarily, a meta-analysis by Brandao and Faciola (2019) reported that continuous cultures produce similar total and individual VFA concentrations and NH_3N concentrations. Although, there are differences between continuous cultures and in vivo trails, continuous cultures offer a faster and cheaper way to evaluate experimental treatments. In contrast to batch cultures, this in vitro technique can remove fermentation end-products and maintain stable conditions (e.g., microbial fermentation) for more extended periods of time.

Ionophores in the Ruminant

Ionophore antibiotics are feed additives that are widely used to improve growth and feed efficiency by changing fermentation patterns in the rumen. Monensin, lasalocid, and laidlomycin propionate are three commercially available ionophores; however, monensin has been used most extensively. Monensin is a carboxylic polyether ionophore that was first introduced as Coban® to control coccidiosis in chickens in 1971. Monensin was later advertised as Rumensin® (Elanco Animal Health, Greenfield, IN), a feed additive that could improve growth and increase feed efficiency by improving energy and protein metabolism in cattle (Bergen and Bates, 1984; McGuffey et al., 2001).

Research has shown that ionophores modify rumen fermentation by inhibiting gram-positive bacteria (Duffield et al., 2008). Gram-positive bacteria lack the outer lipopolysaccharide membrane of gram-negative bacteria, making gram-positive bacteria susceptible to ionophores (Russell and Strobel, 1989). For example, when monensin

penetrates gram-positive bacteria, it can interchange H+ for Na+ or K+ (Russell and Strobel, 1989). Since the potassium gradient is high and the sodium gradient is low inside the bacteria, intracellular K+ will move outside the cell causing extracellular protons to move in the cell (Azzaz et al., 2015). In order to overcome intracellular acidification and depletion of K+, ATPase pumps are activated to eliminate the surplus of protons (Azzaz et al., 2015). However, this depletes the bacterial energy stores, culminating in cellular death. (Russell and Strobel, 1989). As a result, there are less gram-positive bacteria and more gram-negative bacteria.

Ionophores are frequently fed in cattle diets because gram-positive bacteria produce most of the acetate, butyrate, formate, lactate, H_2, and NH_3 found in the rumen (McGuffey et al., 2001). Instead, gram-negative bacteria produce most of the succinate and propionate (McGuffey et al., 2001). Theoretically, if gram-negative bacteria dominate, less CH_4 is produced due to lower H_2 and formate production. The most notable metabolic effects of ionophores on ruminal fermentation are greater propionate production, less acetate, butyrate, lactic acid production, and less dietary protein degradation in the rumen (Bergen and Bates, 1984). As a result of these biological changes, an improvement in energy and nitrogen metabolism has been observed in cattle (Russell and Strobel, 1998; Duffield et al., 2008). Although positive effects may be associated with ionophores, ruminant nutritionists are focused on finding natural alternatives because consumers have an unfavorable perception of antibiotics in food animals because they have concerns about antibiotic resistance and drug residues.

Feed Additive Alternatives

Secondary metabolites have become appealing for ruminant nutritionists because they can exert antimicrobial activities against numerous microorganisms (Kubo et al., 1993). Many plants produce a wide range of secondary metabolites, a class of compounds that are not involved in fundamental plant development but benefit the plant by protecting it from insects and grazing herbivores (Bodas et al., 2008). Typical forms of secondary metabolites are tannins, essential oils, and phenolic compounds; however, thousands have been discovered (Bodas et al., 2008). Although vast, secondary metabolites are not easy to classify because they are the result of multiple and complex metabolic pathways.

Previous research shows that secondary metabolites could be used as alternatives to ionophores because they have been shown to improve rumen metabolism by decreasing acetate and butyrate molar proportions, increasing propionate molar proportions, and by preventing protein degradation in the rumen (Calsamiglia et al., 2007; Patra and Saxena, 2011). These activities could improve energy and protein utilization in ruminants, thereby enhancing their performance. Finding new feed additives, including non-traditional ones, is very relevant and practically crucial to enhance ruminants' efficiency, while addressing public concern on antibiotic use. According to recent literature, tannins, essential oils, and cashew nut shell extract are a few alternatives available for modifying microbial fermentation patterns.

Tannins

Tannins are polyphenol compounds found in various plant species but are widely present in legumes, forages, trees, and shrubs (Kumar and Singh, 1984). Tannins are found in almost every part of the plant and serve to protect it from predation (Dixon et al., 2005). Chemically, the word tannin describes a naturally occurring constituent of high molecular weight containing hydroxylic groups that allow it to bind and precipitate proteins (Kumar and Singh, 1984). Generally, these secondary compounds are divided into two structural groups: hydrolysable tannins and condensed tannins (Kreuger et al., 2009). Hydrolysable tannins (HT) contain a carbohydrate core, which is esterified with phenolic acids (Patra and Saxena, 2011). Since HT are prone to hydrolysis by acids, bases, or esterases, they can be degraded and absorbed in the small intestine (Min et al., 2003). Instead, condensed tannins (CT) are oligomeric or polymeric flavonoid units connected by carbon bonds (Min et al., 2003). Condensed tannins are of interest in ruminant nutrition because of its reactivity with forage proteins after mastication (Min et al., 2003). Condensed tannins forms complexes with carbohydrates (e.g., cellulose and hemicellulose) and protein. In the rumen, CT can bind the protein to form CT-protein complexes (making forage proteins less degradable in the rumen), thereby increasing AA's available for absorption in the small intestine (Min et al., 2003).

The size, chemical structure, and concentration of CT can affect their reactivity, digestion, and nutritional value. Hagerman and Butler (1991) suggests that CT gravitates towards proteins with a greater molecular weight. Kumar and Singh (1984) found that high dietary concentrations (>55 g CT/g DM) can be toxic to ruminants limiting feed

intake, digestibility, and growth. However, lower concentrations of CT can improve body weight, milk yields, and reproductive performance (Min et al., 2003). Dahlberg et al. (1998) reported CT (L. corniculatus) increased total VFA concentration and noted a shift in the acetate to propionate ratio (A:P). Khiasoa-Ard et al. (2009) discovered similar findings with an increase in propionate production, but total VFA production was not affected with supplementation. Landau et al. (2000) described reduced palatability, linking it to the complexes formed between the CT and salivary glycoproteins. Patra and Saxena (2011) reported a decrease in methane produced with the inclusion of CT. Condensed Tannins may have reduced methane production by limiting fiber digestion in the rumen, thus inhibiting the activities of methanogens. Overall, CT has received considerable attention as rumen modifiers, and the effects on ruminants depend on the concentration and structure of the CT.

Essential Oils

Essential oils (EO) are a mixture of secondary metabolites isolated from a variety of plant species (McIntoch et al., 2003). The most active components in EO are terpenoids and phenylpropanoids (Calsamiglia et al., 2007). Terpenoids and phenylpropanoids originate from different precursors (isopentenyl pyrophosphate and phenylalanine, respectively) and are synthesized through different metabolic pathways (Calsamiglia et al., 2007). Essential oils are extremely complex, and their biological actions are strongly influenced by their chemical structure, extraction method, and plant species from which they are extracted (McIntoch et al., 2003).

Research suggests that the primary action of EO as antimicrobials is its activity at the cell membrane (Calsamiglia et al., 2007). Essential oils can alter the structure of bacteria's membranes by accumulating in the lipidic bilayer, causing ions to cross the cell membrane and altering the ionic gradient (Ultee et al., 1999; Griffin et al., 1999). Bacteria can normally offset these effects by employing ion pumps but at the cost of energy, which slows bacterial development (Griffin et al., 1999). Essential oil supplementation has influenced the growth of several bacteria species, including bacteria populations in the rumen (McIntosh et al., 2003; Cardozo et al., 2005; Castiellejos et al., 2006). Therefore, ruminant nutritionists are interested in examining essential oils' potential as rumen fermentation modifiers, specifically, EO impact on energy utilization and ruminal N. Most studies conducted to date have been in vitro using batch culture and continuous culture systems (Joch et al., 2016; Kim et al., 2019). For example, Castillejos et al. (2006) screened different EO's (thymol, eugenol, limonene, guaiacol, and vanillin) in a 60:40 alfalfa hay:concentrate diet. Castillejos et al. (2006) reported thymol supplemented at 50 mg/L had no effect on fermentation profile, but all compounds supplemented at 500 mg/L reduced total VFA and improved final pH. At 50 mg/L eugenol decreased NH_3N concentrations and at 500 mg/L decreased propionate and branch-chain VFAs (Castillejos et al., 2006). Additionally, Castillejos et al. (2006), reported no impact on individual VFAs or NH_3N concentrations with limonene, guaiacol, and vanillin supplementation. Cardozo et al. (2005) evaluated thymol under different dietary conditions (10:90 straw:concentrate) and reported an increase in total VFA concentration and a decrease in the A:P ratio. These results suggest that thymol effects

are heavily influenced by diet and pH (Cardozo et al., 2005; Castillejos et al., 2006). McIntosh et al. (2003) investigated a commercial blend of EO compounds (thymol, eugenol, limonene, and vanillin) and observed a 9% decrease in AA deamination when supplemented at 1 g/cow/d in batch cultures. Newbold et al. (2004) reported a greater reduction in AA deamination (24%) in 24 h incubations. Using the same commercial blend, Benchaar et al. (2007) observed no effect on DM, NDF, acid detergent fiber (ADF), CP, or NH_3N, but reported a decrease in total VFA concentrations when supplemented at 0.75 or 2 g/d in lactating dairy cows. Data from current studies have inconsistent results due to the variation of dosages, biochemical structure of compounds, diets, and EO blends. As a result, more research into the effects of EO on ruminal fermentation, both in vitro and in vivo, are needed before using these compounds to modify microbial populations.

Cashew Tree, Apple, Nut, and Extract

The cashew tree (*Anacardium occidentale*) originates from Brazil, growing naturally in tropical climates (de Brito et al., 2018). It is primarily grown in Asia, Africa, and South America, most notably in Brazil, India, Kenya, Mozambique, and Tanzania (Watanabe et al., 2010). Cashew trees are tropical evergreens that grow to be 10-12 meters tall and produce cashew apples (pseudo-fruit) and cashew nuts (true fruit) (De Lima et al., 2008). Anatomically, the cashew apple emerges from the receptacle of the cashew flower and the apple connects the cashew nut the tree (de Brito et al., 2018; Figure 1.1). When the cashew apple matures into a yellow or red fleshy structure it is used, locally, to make jams, juices, and wines (De Lima et al., 2008). De Lima et al.

(2008) describes the cashew nut as a kidney-shaped structure that is about 2-3 cm long. The cashew nut shell is made up of three layers: the outer shell, the inner shell, and the cashew nut shell liquid (CNSL), which is a vicious liquid located in-between (De Lima et al., 2008). Cashew nut shell liquid is a natural agricultural byproduct of the cashew nut manufacturing process, with a global production of one million t/yr (Lomonaco et al., 2017). This byproduct is known for its industrial uses in pharmaceuticals, cosmetics, adhesives, and insecticides (Kobayashi et al., 2016). In addition, it has been used as a flavor additive in dairy rations in amounts that must not exceed 500 ppm per day (AAFCO, 2019).

Cashew nut shell liquid (CNSL) was initially a broad term used to describe the liquid found between the inner and outer nut shell. Nowadays, the liquid is classified based on the extraction method: (1) cold-pressed extraction or (2) thermal extraction. In literature, CNSL and cashew nut shell extract (CNSE) are interchangeable terms used to indicate mechanical cold-pressed processing. If the liquid is extracted using a thermal process it is called technical-grade cashew nut shell liquid (TCNSL) (Branco et al., 2015). The difference between CNSE and TCNSL are the phenolic constituents present after extraction (Branco et al., 2015). Cashew nut shell extract mainly contains anacardic acids, cardanol, and cardol (Watanabe et al., 2010; AAFCO, 2019). The phenolic compounds present in CNSE must include at least 59% anacardic acid, 18% cardol, and cardanol (AAFCO, 2019). The main constituents of TCNSL are cardanol (63%), and cardol (11%), but TCNSL lacks anacardic acid because of decarboxylation during thermal processing, which changes anacardic acid to cardanol or 2-pentadecadiethyl

phenol (Tyman et al., 1978). Naturally, CNSE contains a substantial amount of anacardic acid, which has piqued the interest of ruminant nutritionists due to its potential to selectively inhibit gram-positive bacteria, which is comparable to the mechanism of action of ionophores on the cell membrane (Kubo et al., 1993). Anacardic acid, according to Van Nevel et at. (1971), may favorably promote the metabolic processes that produce propionate by suppressing methanogenesis. More recent literature proposed the principal activity of anacardic acid and similar phenolics, is a surfactant action that reduces gram-positive bacteria, including bacilli and staphylococci (Watanabe et al., 2010; Oh et al., 2017). Given the abundance of these chemicals in CNSE, it's believed that this product has the ability to improve feed energy and protein utilization in the rumen.

Most studies conducted to date have been in vitro using batch culture, semi-continuous or continuous culture systems; nevertheless, research for its effectiveness as a ruminant feed additive in the United States is scarce. Watanabe et al. (2010) evaluated the impact of cashew nutshell liquid (CNSE) on rumen fermentation in three in vitro experiments: batch culture, semicontinuous culture, and pure cultures. In experiment 1, Watanabe et al. (2010) used batch cultures to screen types of CNSE and TCNSL. After an 18 h incubation, the total volume of headspace was measured using a needle-attached pressure gauge (Aϕ60b, GL Science, Tokyo, Japan). Watanabe et al. (2010) indicated that CNSE, at 500 μg/mL, reduced total gas, CO2, and inhibited methane production by 56.9%, but had no impact on total VFA concentration. However, the molar proportions of propionate were higher, and acetate molar proportions were lower with CNSE supplementation. In experiment 2, Watanabe et al. (2010) added CNSE in stepwise

increments of 0, 50, 100, or 200 μg/mL to assess dose responses using semicontinuous cultures. Watanabe et al. (2010) found that anacardic rich, CNSE decreased methane production (70.1%) and increased propionate production (44.4%) at the highest level (200 μg/mL) of supplementation in semicontinuous cultures. Additionally, CNSE supplementation modified microbial populations by reducing protozoa numbers (from $10^{3.75}$ to $10^{3.24}$) and changing the bacterial species present in the cultures (Watanabe et al., 2010). In experiment 3, pure cultures were used to detect selective inhibition of rumen bacteria by CNSE. Watanabe et al. (2010) reported a decrease in active hydrogen and formate producers (e.g., *R. flavefaciens* and *B. fibrisolvens*) and an increase in active propionate and succinate producers (e.g., *S. dextrinosolvens* and *M. elsdenii*).

Following the encouraging findings of Watanabe et al. (2010), CNSE has been evaluated in other in vitro and in vivo studies. Danielsson et al. (2014) evaluated CNSE supplementation (5 and 10 mg/mL) in a gas in vitro system and reported a decrease in methane production (8 and 18%, respectively). Shinkai et al. (2012) used non-lactating cows to test nutrient digestibility, rumen fermentation, and methane production in two feeding trials. In trial 1, Shinkai et al. (2012) reported a decrease in DMD from 75.6 to 72.3% but observed no effect on NDFD. In trial 2, DMD did not differ and the NDFD tended to increase from 57.9 to 60.8% (Shinkai et al., 2012). Trial 1 and 2 showed increase in molar proportion of propionate and a decrease in methane production per unit of DMI (38.3 and 19.3%, respectively) (Shinkai et al., 2012). Maeda et al. (2021) monitored changes in methane production using respiration chambers and reported a 21.8% decrease in methane emissions with no significant effect on DM, OM, ether

extract (EE), NDF, and gross energy (GE) digestibility when supplementing two doses (4 g of CNSE/100 kg of BW and 6 g of CNSE/100 kg of BW) in beef cattle. Additionally, El-Zaiat et al. (2014) reported no additive influence of CNSE on DM, OM, CP, and EE in growing lambs. These results are consistent with findings reported in a feeding study on periparturient Holstein cows administered 5.0 g of CNSE/head/d (Goetz et al., 2021). Goetz et al., 2021 observed no effect on DM, OM, NDF, ADF, or starch digestibility with CNSE supplementation. Despite the fact that results have varied between in vitro and vivo studies, it appears that CNSE has the potential to reduce methane and increase propionate in ruminants without negative effects on digestibility. As a result, the studies presented in this thesis will continue to assess CNSE's impact on nutrient digestibility and ruminal fermentation in vitro while trying to discover an optimal dose for dairy cows.

Figure 1.1 Cashew apple and cashew nut (seed) (Adapted from Idemitsu Kosan Co. Ltd. (Sodegaura, Japan)).

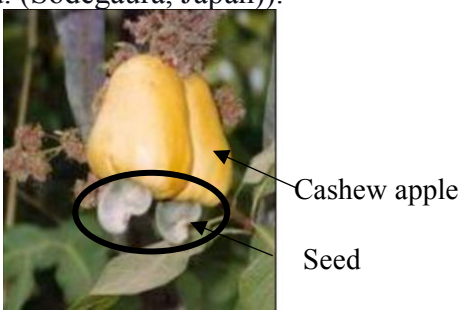
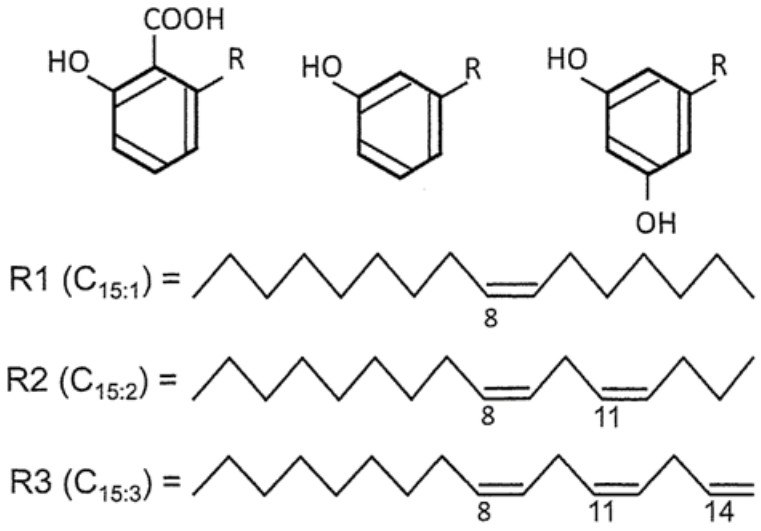


Figure 1.2 Phenolic compounds present in cashew nut shell extract represented by anacardic acid (top left), cardanol (center), and cardol (top right); all salicylic acid derivatives with a C-15 alkyl group (Adapted from Idemitsu Kosan Co. Ltd. (Sodegaura, Japan)).



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CHAPTER TWO

EFFECTS OF CASHEW NUT SHELL EXTRACT ON NUTRIENT DIGESTIBILITY AND RUMINAL FERMENTATION WHEN SIMULATING CLOSE-UP OR FRESH COW CONDITIONS UNDER IN-VITRO BATCH CULTURE CONDITIONS

ABSTRACT

The objective of this study was to determine the effects of cashew nut shell extract (**CNSE**; 59% anacardic acid and 18% cardol) on in vitro dry matter digestibility (**IVDMD**), in vitro true dry matter digestibility (**IVTDMD**), in vitro neutral detergent fiber disappearance (**IVNDFD**), total nitrogen digestibility (**TNd**), and rumen fermentation profile under in vitro batch culture conditions. The study was organized as a randomized complete block design with 15 replicates per treatment incubated for 24-h during four incubation runs (incubation run was the blocking factor). Each incubation was inoculated with rumen contents collected from ruminally fistulated cows fed either a close-up (**CU**; 15.1% CP, 38.1% NDF, and 20.8% starch) or a fresh cow diet (**FC**; 16.4% CP, 31.8% NDF, and 28.0% starch). The CU diet was a high-forage diet fed 3 wk before parturition until calving and FC diet was a high-grain diet fed from calving until 3 wk after parturition. Diets fed to donor cows were the same as the substrate used in the batch culture incubations. Treatments consisted of four levels of granulated CNSE formulated to contain 50% CNSE and added to the cultures in stepwise increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d of CNSE. Data were analyzed with the MIXED procedure of SAS and pre-planned orthogonal contrasts to test for linear and quadratic effects. Overall, dietary treatments incubated to mimic CU conditions had no impact on IVDMD, IVTDMD, IVNDFD, and TNd. Increasing CNSE level decreased the molar proportion of

acetate (quadratic effect), propionate (linear effect), increased isovalerate and valerate, and pH (quadratic effect) in CU diets. Dietary treatments incubated to mimic FC conditions tended to linearly decrease IVDMD (52.4 to 49.5%), IVTDMD (74.6 to 73.6%), IVNDFD (21.6 to 16.7% of DM), and TNd (47.2 to 44.6% of DM). Acetate molar proportions tended to decrease (linear effect), whereas isovalerate and valerate showed a dose-dependent increase (linear effect). When mimicking FC conditions, NH_3N decreased at all levels of supplementation in a linear and quadratic manner. Culture pH increased at 2.5 and 5.0 g/head/d and decreased at 10 g/head/d (quadratic effect). After 24 h of incubation, culture pH was similar for both dietary conditions (6.80 vs. 6.83, respectively). The highest pH (6.88) was observed at 2.5 g/head/d level for CU conditions, and the highest pH (6.87) was observed at 2.5 g/head/d level in FC diets. Under the conditions of this study, adding incremental levels of CNSE had no effects on IVDMD, IVNDFD, or TNd in CU diets but negatively impacted digestibility in FC diets. Further research is warranted to identify the optimal dosage of CNSE.

INTRODUCTION

The transition to lactation is often regarded as the most challenging stage of the lactational cycle for dairy cows (Drackley, 1999). Cows are subjected to multiple metabolic and nutritional changes during this period, making the early lactation cow vulnerable to metabolic disorders. (Cardoso et al., 2020). The nutritional changes during the transition period (3 wk prior to 3 wk following parturition) directly impact microbial populations (Zhu et al., 2018). Various dietary supplements have been used to modify microbial populations to increase the energy supply to the animal during this transition. Energy metabolism can be enhanced with dietary supplements by promoting propionate production while lowering methane production; this is critical when switching from dry cow to lactating diets (McGuffey et al., 2001).

Cashew nut shell extract (CNSE) has attracted a lot of attention due to its biological properties, as it contains antimicrobial compounds such as anacardic acid, cardol, and cardanol (Watanabe et al., 2010; AAFCO, 2019). Anacardic acid is regarded to have the most antimicrobial activity (Kubo et al., 1993). Watanabe et al. (2010) screened CNSE and technical-grade cashew nut shell liquid (i.e., **TCNSL**) and found that CNSE supplementation increased molar proportion of propionate, decreased acetate and butyrate, and reduced methane production by 56.9%. Although TCNSL increased the molar proportion of propionate and lowered acetate, it was to a lower extent (Watanabe et al., 2010). However, Danielsson et al. (2014) found that CNSE added to batch cultures reduced methane production by 18%. According to Branco et al. (2015), TCNSL did not affect methane production and provided no data on VFA concentration. Anacardic acid

and cardanol showed anti-biofouling activity against *Pseudomonas fluorescens*, but anacardic acid killed cells faster (18 h) compared to cardanol (30 h) (Yoon and Kim, 2009). Compared to TCNSL, which is cardanol-rich (<55% cardanol; AAFCO, 2019), anacardic acid appears to better benefit fermentation in the rumen (Yoon and Kim, 2009; Watanabe et al., 2010; Branco et al., 2015).

Although studies have shown that CNSE supplementation can modulate rumen fermentation, little research has been done to see how it affects nutrient digestibility and fermentation during the transition period. Furthermore, before CNSE can be supplemented in dairy cow diets, the balance between an effective and deleterious dose and the potency under different dietary conditions specifically diets commonly used on commercial farms, must be determined. The objective of this study was to evaluate the effects of CNSE (59% anacardic acid and 18% cardol) on nutrient digestibility and rumen fermentation profile during the transition period in close-up and fresh cow diets (i.e., CU and FC, respectively) using an in vitro batch culture system. We hypothesized that incorporating incremental levels of CNSE would not negatively affect nutrient digestibility or rumen fermentation under batch culture conditions.

MATERIALS AND METHODS

Experimental Design and Treatments

The study was organized as a randomized complete block design with 15 replicates per treatment incubated for 24 h during four incubation runs. Treatments consisted of four levels of granulated CNSE formulated to contain 50% CNSE and added to diets in stepwise increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d. For incubation

runs 1 and 2, treatments were added to a CU diet, and for incubation runs 3 and 4, treatments were added to a FC diet. The predicted nutrient composition was determined using NRC (2001). At the start of each incubation run, dietary ingredients were ground through a 2-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA), properly mixed, and 1 g weighed into F57 filter bags (Ankom Technology Corp., Fairport, NY) before being heat-sealed and placed into the flasks. Dietary ingredients and chemical composition are outlined in Table 2.1. Each incubation was inoculated with rumen contents collected from ruminally fistulated cows fed either a CU diet or FC diet.

Rumen Fluid Collection and Flask Conditions

The Clemson University Institutional Animal Care and Use Committee approved all surgical and animal care protocols (DF-AH-015v2). Before rumen fluid collection, the Erlenmeyer glass flasks (125 mL) and butyl rubber stoppers were cleaned and assembled. Approximately 2-3 weeks pre-calving, ruminal contents were collected from three rumen fistulated cows fed a CU diet. Similarly, two of the same fistulated cows fed an FC diet were used as rumen fluid donors 2-3 weeks after calving. Following parturition, one cow from the CU group died. Diets fed to donor cows were the same as the substrate used in the batch cultures. After collection, the solid particles were removed by straining ruminal contents through two layers of cheesecloth. The rumen fluid from all cows was combined and transported to the fermentation laboratory using a prewarmed insulated container. During rumen fluid collection, the flasks were maintained at 39°C in a water bath to maintain an adequate temperature for inoculation. The strained rumen fluid was combined with a medium in a 1:4 ratio and purged with CO_2 until transferred into the

Erlenmeyer flasks. F57 filter bags (Ankom Technology Corp., Fairport, NY) were pre-prepared with 1 g of ration were added to the flasks. Each flask was inoculated with 100 mL of diluted inoculum (20 mL inoculum and 80 mL medium) and placed in a 39°C shaker water bath (70 rpm; Julabo SW22, Seelbach, Germany) for 24 h of incubation. Anaerobic conditions were maintained by purging the headspace of flask with CO_2 until it was sealed with a butyl rubber stopper. Within 60 min, the rumen contents were collected, diluted, and added to the flasks.

Sample Collection and Analysis

After 24 h of incubation, bags were removed from the flasks, rinsed with distilled water, and gently squeezed to eliminate excess water and gas. The filter bags were then dried for 48 h at 55°C (forced-air oven) to determine the apparent in vitro DM digestibility (IVDMD). After weighing the undigested residue, the single-stage IVDMD was calculated by using the following equation [1].

$$\text{IVDMD}_{(\%)} = \left(\frac{\text{Initial DM}_{(g)} - \text{IV DM Residue}_{(g)}}{\text{Initial DM}_{(g)}}\right) \times 100 \quad [1]$$

After determining IVDMD, filter bags were placed in the Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NW) to determine in vitro true DM digestibility (IVDMTD) and in vitro NDF disappearance (IVNDFD). To determine true digestibility, any remaining soluble fractions (i.e., endogenous sources) must be removed using a neutral detergent solution. Following the procedures by Van Soest et al. (1991), sodium sulfate and a heat resistant α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Louis,

MO) were used for NDF analysis, and IVTDMD was determined following further extraction with neutral detergent as described by equation [2]. In addition, IVNDFD was calculated using equation [3].

$$\text{IVTDMD}_{(\%)} = \left(\frac{\text{Initial DM}_{(g)} - \text{IV NDF Residue}_{(g)}}{\text{Initial DM}_{(g)}}\right) \times 100 \quad [2]$$

$$\text{IVNDFD}_{(\%)} = \left(1 - \frac{100 - \text{IVDMTD}_{(\%)}}{\text{NDF}_{(\%)}}\right) \times 100 \quad [3]$$

Culture contents were mixed thoroughly in the Erlenmeyer flasks before sampling to ensure adequate representation of contents. Each culture's pH was measured and recorded following the 24 h incubation. A 5 mL sample of mixed culture contents was pipetted into 15 mL polycarbonate centrifuge tubes containing 1 mL of metaphosphoric acid (25%, w/v) and frozen at -20°C until further analysis (Moody et al., 2007). Samples were thawed and centrifuged at 40,000 x g for 20 min at 4ºC. Following centrifugation, 1 mL of the supernatant was transferred into a 1.5 mL Eppendorf microcentrifuge tube for analysis of NH_3N with reduced sample and reagent volumes (96-well plate reader) as described by Chaney and Marbach (1962). Another 0.5 mL of the supernatant was filtered, diluted with 0.5 mL dH_2O, and combined with 100 µL of internal standard (86 µmol of 2 ethylbutyric acid/mL), in a 2 mL GC vial. The samples were injected into a Hewlett-Packard 6980 gas chromatograph (San Jose, CA) fitted with a custom packed column for VFA-flame-ionization detection (Yang and Varga, 1989).

Feed samples were ground through a 2-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) and evaluated for analytical DM at 100°C for 24 h In order to calculate ash concentration, samples were combusted in a furnace for 3 h at 600°C (AOAC, 2006). Following the procedures by Van Soest et al. (1991), sodium sulfate and a heat resistant α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Louis, MO) were used for NDF analysis, but not ADF. Cumberland Valley Analytical Services analyzed a subsample for total nitrogen (TN) (Leco FP-2000 Nitrogen Analyzer, Leco Instruments Inc., St. Joseph, MI). Crude protein concentration was expressed as percentage N × 6.25.

Statistical Analysis

Data were analyzed with the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) as a randomized complete block design with treatment (dose level) as a fixed effect, and incubation run (1 to 2) and water bath (1 to 4) as random effects, using the following model:

$$Y_{ijk} = \mu + T_i + W_j + R_k + e_{ijk}$$

Where Y_{ijk} is the dependent variable, μ the overall mean, T_i is the fixed effect of treatment, W_j is the random effect of water bath, R_k is the random effect of incubation run, and e_{ijk} as the residual error assumed to be independent and normally distributed (Shapiro-Wilk test). The null hypothesis of normality was rejected ($P < 0.05$ and small Shapiro-Wilk statistic) for NH_3N in CU diets and total VFA in FC diets. Therefore, this data was log-transformed and analyzed statistically. Pre-planned orthogonal contrasts were used to test linear and quadratic effects of treatment. Statistical significance was

declared for $P \leq 0.05$ and tendencies were considered at a value of $P \leq 0.10$. Close-up and FC incubations were analyzed separately, but with the same model.

RESULTS

Diet Composition

Ingredient and chemical composition values of CU and FC diets are presented in Table 2.1. Diets were formulated to represent a CU and a FC diet and different premixes were supplemented during the CU (26.2 % of DM) and FC (51.9 % of DM) periods. SoyChlor and calcium carbonate were added in the CU diet (7.6 and 1.4 % of DM, respectively), but were not added to the FC diet. The dietary CP, starch, and NFC were lower for CU diets compared to the FC diets, whereas the NDF was higher for the CU diets. As the demands for glucose, AA, and fatty acids dramatically increase after parturition, the forage to concentrate ratio is typically altered to favor concentrates (Zhu et al., 2018).

Apparent Digestibility of Nutrients

Table 2.2 shows the effects of increasing concentrations of CNSE (0, 2.5, 5.0, and 10.0 g/head/d) on apparent digestibility from CU incubations. When mimicking CU conditions, adding incremental levels of CNSE had no effect on IVDMD (40.0% ± 4.9), IVTDMD (69.0% ± 2.3), IVNDFD (18.4% ± 6.2), and TNd (32.0% ± 3.0).

Table 2.3 shows the effects of increasing concentrations of CNSE (0, 2.5, 5.0, and 10.0 g/head/d) on apparent digestibility from FC incubations. When simulating FC conditions, adding incremental levels of CNSE tended to linearly decrease IVDMD (52.4

to 49.5%), IVTDMD (74.6 to 73.6%), IVNDFD (21.6 to 16.7% of DM), and TNd (47.2 to 44.6% of DM).

Characteristics of Fermentation

Culture VFA profile, pH, and NH_3N from CU incubations are presented in Table 2.4. Adding incremental levels of CNSE had no effect on molar proportions total VFA, isobutyrate, and butyrate or A:P ratio, but decreased the molar proportions of propionate (linear effect) and acetate (linear and quadratic effect), and increased isovalerate and valerate molar proportions. Culture pH increased with CNSE supplementation, whereas highest pH (6.88) was observed at 2.5 g/head/d level and the lowest pH (6.75) was observed at 10.0 g/head/d level (quadratic effect). No difference was found between dose levels on NH_3N production under CU conditions.

Culture VFA profile, pH, and NH_3N from FC incubations are presented in Table 2.5. Overall, adding incremental levels of CNSE had no impact on total VFA concentrations, propionate, isobutyrate, and butyrate molar proportions. Acetate molar proportions tended to decrease (linear effect), whereas isovalerate and valerate showed an increase (linear effect) with supplementation. Culture pH increased at 2.5 and 5.0 g/head/d and decreased at 10 g/head/d (linear and quadratic effect) compared to the control. Ammonia nitrogen decreased at all levels of supplemented CNSE in a linear and quadratic manner ($P = 0.01$).

DISCUSSION

Close-up Mimicking Conditions

Under the CU conditions of this study, increasing the level of CNSE had no linear or quadratic effect on IVDMD, IVTDMD, IVNDFD, or TNd. Compared to previous research, increasing the level of CNSE in this study resulted in similar nutrient digestibility responses. For example, these results are consistent with findings reported in a feeding study on periparturient Holstein cows administered 5.0 g of CNSE/head/d (Goetz et al., 2021). Goetz et al., 2021 observed no effect on DM, OM, NDF, ADF, or starch digestibility with CNSE supplementation. Similarly, CNSE administered at a much higher dose (4 g of CNSE/100 kg of BW and 6 g of CNSE/100 kg of BW) did not affect DM, OM, EE, NDF, or GE digestibility in beef cattle (Maeda et al., 2021). Shinkai et al. (2012) used non-lactating cows to test nutrient digestibility in two feeding trials, each with a total intake of 4 g of CNSE/100 kg of BW. Yet, changes in nutrient digestibility were inconsistent between trials. In trial 1, Shinkai et al. (2012) reported a decrease in DMD from 75.6 to 72.3% but observed no effect on NDFD with supplemented CNSE. In trial 2, DMD did not differ and the NDFD tended to increase from 57.9 to 60.8% (Shinkai et al., 2012). Shinkai et al. (2012) suggest that differences in nutrient digestibility may have been primarily influenced by pellet composition, as the pellet fed in trial 1 contained only silica powder, but the pellet fed in trial 2 contained numerous ingredients (e.g., alfalfa meal, defatted rice bean, silica powder, molasses, and tapioca flour). The authors did not discuss the effect of pelleting on phenolic acid profiles, but pelleting produces heat, which converts anacardic acid to cardanol (Tyman et al., 1978).

The impacts of CNSE supplementation on nutrient digestibility have not been thoroughly explored in vitro or in vivo; however, administering CNSE to dry cows does not appear to negatively impact nutrient digestibility.

Under the CU conditions of this study, CNSE supplementation did not influence the molar proportion of total VFA. Similarly, Watanabe et al. (2010) observed no effect on the molar proportion of total VFA in batch cultures but found that total VFA concentration increased quadratically ($P = 0.05$) under semicontinuous conditions. Other effects of CNSE on ruminal fermentation were the dose-dependent decrease (linear and quadratic effect) in molar proportion of acetate (Table 2.4), decrease (linear effect) in molar proportion of propionate, increase in isovalerate and valerate, and no significant change in the molar proportion of isobutyrate and butyrate. A decrease in the molar proportion of acetate was consistent with results from an in vitro study by Watanabe et al. (2010) and an in vivo study by Shinkai et al. (2012). Furthermore, Watanabe et al. (2010) reported a decrease in active hydrogen and formate producers (e.g., *R. flavefaciens* and *B. fibrisolvens*) and an increase in active propionate and succinate producers (e.g., *S. dextrinosolvens* and *M. elsdenii*). Based on these findings, the authors suggest CNSE supplementation may inhibit certain gram-positive bacteria, while indirectly promoting certain gram-negative bacteria (Watanabe et al., 2010). However, in the present study, the effect of CNSE on ruminal propionate and butyrate are inconsistent with previous studies (Watanabe et al., 2010; Shinkai et al., 2012). Watanabe et al. (2010) reported a significant increase (linear effect) in molar proportion of propionate and a decrease (quadratic effect) in butyrate in vitro. Shinkai et al. (2012) confirmed the increase in molar proportion (but

not concentration) of propionate in vivo. Similar results were found with monensin, which alters the proportions of VFA towards higher propionate and lower acetate production in the rumen under in vitro and in vivo studies (Richardson et al., 1976). Yet, this result could not be confirmed in the present study. Ruminal pH increased at 2.5 and 5.0 g/head/d and decreased at 10 g/head/d in a linear ($P < 0.05$) and quadratic manner ($P < 0.02$). Whereas highest pH (6.88) was observed at 2.5 g/head/d level, and the lowest pH (6.75) was observed at 10.0 g/head/d level. However, Watanabe et al. (2010) reported a quadratic decrease in ruminal pH at all levels of CNSE supplementation (50, 100, and 200 μg/mL) in vitro. In contrast, Shinkai et al. (2012) reported no change in ruminal pH in vivo. Ammonia nitrogen concentration was unaffected as CNSE increased in CU diets. Similarly, Shinkai et al. (2012) reported no change in NH_3N concentrations when supplementing dry cows at 4 g of CNSE/100 kg of BW; however, NH_3N decreased quadratically ($P < 0.04$) with 50, 100, and 200 μg/mL of CNSE supplementation under semicontinuous culture conditions (Watanabe et al., 2010).

Fresh Period Mimicking Conditions

Under the FC conditions of this study, IVDMD, IVTDMD, IVNDFD, and TNd tended to decrease linearly ($P < 0.10$) (Table 2.3). These results suggest that gram-positive bacteria may be more sensitive to CNSE, but molar proportions of individual VFA and A:P ratio in this experiment do not wholly support this theory. In addition, these findings are inconsistent with results reported in postpartum Holstein cows administered 2.5 and 5.0 g/head/d of CNSE (Goetz et al., 2021). Goetz et al. (2021) observed no effect on DM, OM, NDF, ADF, or starch digestibility with CNSE

supplementation. Effects of administering CNSE on nutrient digestibility using lactating cows has been previously evaluated with TCNSL that contains cardanol as the main phenolic compound (Coutinho et al., 2014; Branco et al., 2015). Coutinho et al. (2014) reported TCNSL supplemented at 7 g/head/d had no effect on DMI or apparent total tract digestibility. Similarly, Branco et al. (2015) observed no effect on apparent total tract digestibility (DM, OM, CP, or ADF), but NDF digestibility tended to increase compared with no supplementation. One limitation of most available data is that current experiments evaluate the nutrient digestibility using TCNSL (73.3% cardanol, 16.4% cardol, and 3.0% methylcardol (Branco et al., 2015). Technical-grade cashew nut shell liquid lacks the most potent antimicrobial compound, anacardic acid (Yoon and Kim, 2009; Watanabe et al., 2010). It is important to note that although TCNSL and CNSL contain similar compounds, the proportions in which they are represented differ significantly, thus microbial response may be differentially impacted.

Under the FC conditions of this study, CNSE supplementation did not affect total VFA molar proportion. Similarly, Watanabe et al. (2010) observed no effect on molar proportion of total VFA in batch cultures. Oh et al. (2017) evaluated CNSE under 5 dietary conditions with varying forage to concentrate (F:C) ratios (9:1, 7:3, 5:5, 3:7, and 1:9) in batch cultures. The authors reported no change in molar proportion of VFA with CNSE supplementation in 4 of the 5 experimental diets, except for 5:5 F:C, which increased the molar proportion of total VFA (Oh et al., 2017). In this study, acetate molar proportion tended to be lower with higher CNSE levels ($P < 0.07$; Table 2.5), but the opposite was observed for isovalerate and valerate. Although a trend was observed,

acetate decreased significantly with supplementation in previous in vitro and in vivo studies (Watanabe et al., 2010; Shinkai et al., 2012; Danielsson et al., 2014; Oh et al., 2017). When mimicking FC conditions, propionate and butyrate molar proportions, and the A:P ratio was not affected by CNSE levels. However, these results are inconsistent with previous findings (Watanabe et al., 2010; Shinkai et al., 2012; Danielsson et al., 2014; Oh et al., 2017). Watanabe et al. (2010) reported a significant increase (linear effect) in molar proportion of propionate and decrease (quadratic effect) in molar proportion of butyrate in batch cultures. Additionally, Oh et al. (2017) reported a significant increase in propionate and decrease in butyrate with addition of CNSE. Shinkai et al. (2012) confirmed the increase in molar proportion (but not concentration) of propionate in vivo. Ruminal pH numerically increased at 2.5 and 5.0 g/head/d and decreased at 10 g/head/d and a linear tendency ($P < 0.07$) and quadratic response ($P < 0.02$) was observed. Highest pH (6.87) was observed at 2.5 g/head/d level and the lowest pH (6.76) was observed at 10.0 g/head/d level. These findings are similar to results reported for CU conditions, where the highest pH (6.88) was observed at 2.5 g/head/d level and the lowest pH (6.75) was observed at 10.0 g/head/d level. Ruminal pH was numerically similar for both dietary conditions (6.80 vs. 6.83, respectively) because the medium supplied in the study kept the culture around the same pH level over the 24 h incubation period. Ammonia concentration decreased linearly and had a quadratic response as CNSE increased in the FC diets (Table 2.5). Reductions in ruminal ammonia concentration with CNSE supplementation have reportedly varied. Ammonia concentrations changed quadratically ($P < 0.04$) with 50, 100, and 200 µg/mL of CNSE

with the greatest reduction at 200 μg/mL level of supplementation in vitro (Watanabe et al., 2010). Likewise, Goetz et al. (2020) reported a decrease in NH_3N with CNSE supplementation in early lactation cows. These results imply that CNSE supplementation could inhibit proteolytic bacteria or promote ammonia uptake (Watanabe et al., 2010). In contrast, Shinkai et al. (2012) reported no effect to NH_3N with CNSE supplementation.

Although CU and FC incubations were not compared statistically, there was a numerical difference in the A:P ratio (2.60 vs. 2.04, respectively). As expected, acetate molar proportion was higher, propionate molar proportion was lower, and the A:P was higher for CU incubations compared to FC incubations. Differences in A:P ratio indicate the nature of rumen fermentation because: (1) changes in starch contents and intake can shift individual VFA concentrations (2) increasing the amount of starch in the diet reduces acetate production while increasing propionate production in the rumen (Manthey and Anderson, 2018). As a result, the batch culture system demonstrated a shift in VFA profile between the CU and FC diets after 24 h of incubation.

CONCLUSIONS

Under the conditions of this experiment, adding incremental levels of CNSE showed different effects on nutrient digestibility and culture fermentation in CU and FC diets after 24 h incubations. Within the range of doses from this study, CNSE had no detrimental impact on nutrient digestibility under CU conditions but may negatively impact digestibility under FC conditions. Specifically, CNSE had no detectable effect on nutrient digestibility in CU diets but tended to decrease digestibility in FC diets with increasing levels of CNSE. Acetate and propionate molar proportions decreased in CU runs and acetate molar proportion tended to decrease in FC runs. Rumen pH after 24 h of incubation was similar for both dietary conditions (6.80 vs. 6.83, respectively), and a quadratic response was observed when mimicking both CU and FC conditions. Ammonia nitrogen concentration was unaffected as CNSE increased in CU diets, but linearly and quadratically decreased in FC diets. The advantages of CNSE supplementation should be further assessed because CNSE showed the potential to decrease acetate in both CU and FC diets without negatively impacting nutrient digestibility in CU diets.

Table 2.1 Ingredient and chemical composition of close-up (CU) and fresh cow (FC) diets containing different levels of cashew nut shell extract (CNSE; 0, 2.5, 5.0, 10.0 g/head/d) fed to in vitro batch culture system.

	CU				FC			
	CNSE (g/head/d)				CNSE (g/head/d)			
	0	2.5	5.0	10.0	0	2.5	5.0	10.0
Ingredient[1]								
Corn Silage	51.6	51.6	51.6	51.6	44.0	44.0	44.0	44.0
Barley baleage	5.2	5.2	5.2	5.2	4.1	4.1	4.1	4.1
Bermuda hay	7.8	7.8	7.8	7.8	23.0	23.0	23.0	23.0
Dry cow premix[2]	26.2	26.2	26.2	26.2	--	--	--	--
Lactating premix[3]	--	--	--	--	51.9	51.9	51.9	51.9
SoyChlor	7.6	7.6	7.6	7.6	--	--	--	--
Calcium carbonate	1.4	1.4	1.4	1.4	--	--	--	--
Chemical composition								
CP, % DM	15.1	15.1	15.1	15.1	16.4	16.4	16.4	16.4
NDF, % DM	36.6	38.3	39.7	37.9	32.3	31.4	31.3	32.0
Starch, % DM	20.8	20.8	20.8	20.8	28.0	28.0	28.0	28.0
NFC, % DM	33.4	33.4	33.4	33.4	38.9	38.9	38.9	38.9

[1]All diets were ground to 2 mm.

[2]Ingredient composition (% DM) for dry cow premix: soybean meal 26.2%, soy chlor 22.3%, soybean hull 17.8%, soyplus 14.0%, calcium carbonate 9.4%, corn 5.6%, citrus pulp 5.0%, magnesium sulfate 0.9%, calcium phosphate 0.9%, salt 0.7%, vitamin E 0.4%, dairy Vt250 0.3%, magnesium oxide 0.2%, selenium 0.1%.

[3]Ingredient composition (% DM) for fresh cow premix: corn, ground 31.4%, soybean meal 18.2%, soyplus 16.9%, corn gluten 11.8%, soybean hulls 4.7%, whole cotton 5.1%, calcium carbonate 2.9%, molasses 2.3%, sodium bicarbonate 2.2%, bentonite 0.8%, palmit 80 0.7%, magnesium oxide 0.6%, salt 0.6%, potassium carbonate 0.5%, potassium chloride 0.5%, urea 0.4%, dairy VTM250 0.2%, vitamin E 20,000 0.1%.

Table 2.2 Effect of different levels of cashew nut shell extract (CNSE) supplementation (0, 2.5, 5.0, 10.0 g/head/d) on nutrient apparent digestibility in a close-up diet under in vitro batch culture conditions.

	CNSE (g/head/day)					*P* value[1]	
Item	0	2.5	5.0	10.0	SE	L	Q
IVDMD[2], %	41.6	37.8	38.9	40.2	3.22	0.94	0.16
IVTDMD[3], %	69.4	67.9	68.7	69.8	1.34	0.48	0.21
IVNDFD, % DM	16.1	16.1	21.0	20.3	3.51	0.14	0.53
TN, % DM	32.9	32.5	30.5	32.1	2.08	0.54	0.24

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of increasing CNSE supplementation level.
[2]IVDMD = in vitro 24-h dry matter digestibility.
[3]IVTDMD = in vitro 24-h true dry matter digestibility.

Table 2.3 Effect of different levels of cashew nut shell extract (CNSE) supplementation (0, 2.5, 5.0, 10.0 g/head/d) on nutrient apparent digestibility in a fresh cow diet under in vitro batch culture conditions.

	CNSE (g/head/day)					*P* value[1]	
Item	0	2.5	5.0	10.0	SE	L	Q
IVDMD[2], %	52.4	51.2	50.8	49.5	1.95	0.08	0.81
IVTDMD[3], %	74.6	74.3	73.7	73.6	0.92	0.10	0.76
IVNDFD, % DM	21.6	18.4	16.0	16.7	2.90	0.08	0.18
TN, % DM	47.2	46.2	47.0	44.6	1.72	0.09	0.58

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of increasing CNSE levels.
[2]IVDMD = in vitro 24-h dry matter digestibility.
[3]IVTDMD = in vitro 24-h true dry matter digestibility.

Table 2.4 Effect of different levels of cashew nut shell extract (CNSE) supplementation (0, 2.5, 5.0, 10.0 g/head/d) on volatile fatty acids (VFA), pH, and ammonia nitrogen (NH_3N) concentration in a close-up diet under in vitro batch culture conditions.

	CNSE (g/head/d)					*P* value[1]	
Culture fermentation	0	2.5	5.0	10.0	SE	L	Q
Total VFA, mM	47.2	45.5	50.8	44.2	11.3	0.42	0.13
VFA, mol/100 mol							
Acetate	57.1	57.0	54.0	55.0	4.17	<0.01	0.05
Propionate	21.9	21.9	21.2	21.3	0.33	0.02	0.50
Isobutyrate	3.10	2.77	3.44	3.58	2.25	0.35	0.82
Butyrate	11.5	11.4	12.1	11.7	2.99	0.33	0.24
Isovalerate	4.01	4.01	4.49	4.40	1.22	<0.01	0.20
Valerate	2.23	2.63	3.64	3.51	1.46	<0.01	<0.01
A:P[2]	2.62	2.61	2.56	2.60	0.22	0.64	0.49
pH	6.78	6.88	6.80	6.75	0.03	0.05	0.02
NH_3N, mg/dL[3]	13.7	15.4	10.7	12.5	2.29	0.21	0.26

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of increasing CNSE levels.

[2]Acetate to propionate ratio.

[3]Statistical analysis was performed on log-transformed data.

Table 2.5 Effect of different levels of cashew but shell extract (CNSE) supplementation (0, 2.5, 5.0, 10.0 g/head/d) on volatile fatty acids (VFA), pH, and ammonia nitrogen (NH_3N) concentration in a fresh cow diet under in vitro batch culture conditions.

	CNSE (g/head/d)					*P* value[1]	
Culture fermentation	0	2.5	5.0	10.0	SE	L	Q
Total VFA, mM[2]	57.0	49.1	51.1	51.9	10.7	0.81	0.39
VFA, mol/100 mol							
Acetate	53.2	52.3	52.3	52.0	1.49	0.07	0.36
Propionate	25.9	26.3	25.7	25.9	1.08	0.51	0.93
Isobutyrate	0.48	0.47	0.48	0.46	0.47	0.72	0.94
Butyrate	12.4	12.5	12.4	12.3	0.40	0.73	0.87
Isovalerate	4.58	4.72	4.92	4.99	0.12	0.01	0.38
Valerate	3.48	3.97	4.21	4.45	0.29	<0.01	0.15
A:P[3]	2.07	2.00	2.05	2.02	0.14	0.43	0.62
pH	6.81	6.87	6.86	6.76	0.27	0.07	0.02
NH_3N, mg/dL	11.4	9.9	9.0	9.9	1.43	0.01	0.01

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of increasing CNSE levels.
[2]Statistical analysis was performed on log-transformed data.
[3]Acetate to propionate ratio.

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CHAPTER THREE

EFFECTS OF CASHEW NUT SHELL EXTRACT ON NUTRIENT DIGESTIBILITY AND RUMINAL FERMENTATION UNDER IN-VITRO CONTINUOUS CULTURE CONDITIONS

ABSTRACT

Various feed additives are in widespread use in ruminants' diets to modulate rumen fermentation, thus improving nutrient utilization and animal performance. The objective of this study was to determine the effects of cashew nutshell extract (**CNSE**, 59% anacardic acid and 18% cardol) on apparent digestibility of dry matter (**DM**), organic matter (**OM**), neutral detergent fiber (**NDF**), total nitrogen (TN), and rumen fermentation profile in continuous culture fermenters. We hypothesized that incorporating incremental levels of CNSE would not negatively affect nutrient digestibility. Treatments consisted of four doses of granulated CNSE formulated to contain 50% CNSE, premixed with corn grain, and added to diets in stepwise increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d. Treatments were randomly assigned to eight fermenters over two 10 d periods. Fermenters were fed 56.2 g/d of TMR (17.0% CP, 29.7% NDF, and 29.9% starch), divided between 2 feedings at 0800 and 2000 h. Each fermenter was inoculated with rumen contents collected from two ruminally fistulated cows in mid-lactation and diluted 1:1 with buffer on day 0. Data were analyzed with the mixed procedure of SAS as a randomized complete block design using pre-planned orthogonal contracts to test for linear and quadratic effects. Increasing levels of CNSE had no effect on DM (54.2 to 56.2%), OM (63.3 to 65.5%) and NDF (60.2 to 62.2%). Total VFA, acetate, propionate, butyrate molar proportions, and the A:P ratio, as well as culture pH, E*h*, and rH, were unaffected by CNSE supplementation

levels. These results suggest that incremental levels of CNSE have no impact on nutrient digestibility and rumen fermentation profile under continuous culture conditions.

INTRODUCTION

Ionophore antibiotics are feed additives that are widely used to improve growth and feed efficiency by changing fermentation patterns in the rumen (Bergen and Bates, 1984; Russell and Strobel, 1988). Consumers, on the other hand, have an unfavorable perception of antibiotics in food animals due to the concerns over antibiotic resistance and drug residues (Greathead, 2003). As a result, many countries have placed restrictions on the use of antibiotics in feed animals, and the European Union has even outlawed them. Therefore, ruminant nutritionists are becoming increasingly interested in using natural alternatives to replace antibiotics to lessen worries about human health risks (Calsamiglia et al., 2007).

Cashew nut shell extract could be an alternative to ionophores and CNSE is more likely to be accepted by consumers. Anacardic acid, cardol, and cardanol are the primary phenolic compounds found in this extract (Watanabe et al., 2010; AAFCO, 2019). The main phenolic compound is anacardic acid, making up at least 59% of the final product (AAFCO, 2019). Like ionophores, antimicrobial properties of anacardic acid may inhibit certain gram-positive rumen bacteria (Kubo et al., 1993). Anacardic acid, according to Van Nevel et at. (1971), may promote the metabolic processes that produce propionate. Watanabe et al. (2010) found that anacardic rich, CNSE decreased methane production (70.1%) and increased propionate production (44.4%) at the highest level (200 μg/mL) of supplementation in semicontinuous cultures. Similar microbial shifts have been reported in vivo (Shinkai et al., 2012). This shift in rumen fermentation is a way to improve the efficiency of feed energy utilization in cows.

The objective of this study was to determine the effects of CNSE (59% anacardic acid and 18% cardol) on apparent digestibility of DM, OM, NDF, TN, culture pH, and molar proportions of total and individual VFA in continuous culture fermenters. We hypothesized that incorporating incremental levels of CNSE would not affect nutrient digestibility or VFA concentrations under continuous culture conditions.

MATERIALS AND METHODS

Experimental Design and Treatments

The study was organized as a randomized complete block design with four treatments. To eliminate fermenter-specific differences, treatments were randomly assigned to one of eight continuous culture fermenters and allocated to a different fermenter each period. Treatments consisted of four doses of granulated CNSE formulated to contain 50% CNSE and added to the diets in stepwise increments equivalent to 0, 2.5, 5.0, and 10.0 g/head/d. A total of 20 d of fermentation were distributed among 2 replicated periods of 10 d each, the first 7 d for adaption and the last 3 d for sample collection. Fuentes et al. (2009) suggests a minimum of 5 d of adaption to stabilize the microbial population within the cultures. Fermenters were fed 56.2 g/d of total mixed ration (TMR) and predicted nutrient composition was calculated using NRC (2001). Dietary ingredients and chemical composition are outlined in Table 3.1. Diets were prepared and mixed in advance using a commercial KitchenAid, divided between 2 feedings at 0800 and 2000 h.

Rumen Fluid Collection and Continuous Culture Conditions

The Clemson University Institutional Animal Care and Use Committee approved all surgical and animal care protocols (DF-AH-015v2). On day 0, the fermenters were cleaned and assembled. At approximately 1900 h, ruminal contents were collected from two ruminally fistulated multiparnipous mid-lactation cows fed a comparable diet to that of the fermenters. After collection, the solid particles were removed by straining the ruminal contents through two layers of cheesecloth. The premixed rumen fluid was transported to the laboratory using a prewarmed insulated container. The rumen fluid was combined with buffer in a 1:1 ratio and purged with CO_2 before being supplied to the fermenters (Slyter et al., 1966). Fermenters were purged with CO_2, and the temperature was maintained at 39ºC by a recirculating water bath before inoculation. The diluted inoculum was added to fill each continuous culture fermenter (approximately 800 mL). Within 60 min, the rumen contents were collected, diluted, and added to the fermenters. The design and operation of the cultures were based on Teather and Sauer's (1988) technique, with certain alterations. The key enhancements were a 45° overflow port to assist rumen overflow and a quicker stirring rate (45 rpm) that permitted particle stratification (Jenkins et al., 2014; Lascano et al., 2016). The buffer solution was continuously infused into the culture using a peristaltic pump to achieve a set flow rate of 90 mL/h to maintain a 10-12% liquid dilution rate. Each morning around 0730 h, buffer solution was prepared and adjusted using 6 N NaOH or 3 N HCL to maintain buffer pH levels. All fermenters received the same buffer solution, thus treatment effects of CNSE had the opportunity to alter pH. Anaerobic conditions were maintained by purging the

cultures with CO_2 at a rate of 20 mL/min and were checked nightly to verify consistency. The pH of the culture was continually monitored using pre-installed pH probes and data-logging software was set to measure pH every 20 min. To ensure accuracy, a portable probe was utilized every day. At the beginning of each period, the portable and continuous probes were calibrated.

Sample Collection and Analysis

Beginning on d 7 at 2000 h, the eight 2 L Erlenmeyer flasks used to collect the overflow were submerged in an ice bath, and 10 mL of H_2SO_4 (50% solution) was added inside to prevent further microbial activity. This procedure was repeated on d 8, 9, and 10 at 0800 and 2000 h (excluding d 10 2000 h) after the overflow was collected from each fermenter. The volume of the overflow flask was measured twice daily, at 0800 and 2000 h, and the total daily volume was calculated. At 0800 and 2000 h, 20% of the overflow (200 mL) was collected in a prelabeled container and frozen at -20ºC. Later, the 3 d composited overflow samples were thawed, homogenized, and subsampled (400 mL) for DM, OM, N, and NDF analyses. On the last day (d 10), culture contents were mixed thoroughly (100 rpm) during sampling to ensure proper mixing. At 0 (before feeding), 2, 4, 6, 8, 10, and 12 h after feeding, the pH and oxidation-reduction potential (E*h*) of the culture were measured and recorded. The redox probe (Traceable 4277 pH/ORP Meter, Control Company, Webster, TX) was used to determine E*h*, and the Clark equation was used to calculate the relative hydrogen score (rH) from E*h* and pH (Huang et al., 2018).

Feed samples were ground through a 1-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) and evaluated for analytical DM (at 100°C for 24 h), OM,

NDF and ADF. In order to calculate ash concentration, feed samples were combusted in a furnace for 2 h at 600°C (AOAC, 2006). 200 mL of subsampled composited overflow was transferred equally into DM tubes and centrifuged in an SA600/SS-34 rotor at 16,500 rpm (40,000 G) for 20 min at 4ºC. Once centrifuged, the supernatant was carefully removed using 3 mL plastic transfer pipettes, and the drained DM tubes were immediately dried at for 48 h at 100ºC. Dried overflow samples were ground through a 1-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) and evaluated for DM, OM, and NDF. ANKOM200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY) was used to analyze NDF and ADF. Following the procedures by Van Soest et al. (1991), sodium sulfate and a heat resistant α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Louis, MO) were used for NDF analysis. Cumberland Valley Analytical Services analyzed a subsample for TN (Leco FP-2000 Nitrogen Analyzer, Leco Instruments Inc., St. Joseph, MI). Crude protein concentration was expressed as percentage N × 6.25. In order to calculate ash concentration, samples were combusted in a furnace for 2 h at 600°C (AOAC, 2006), and overflow samples were corrected by ash concentration. A 5 mL sample of mixed culture contents was pipetted into 15 mL polycarbonate centrifuge tubes containing 1 mL of metaphosphoric acid (25%, w/v) and frozen at -20°C until further analysis (Moody et al., 2007). Samples were thawed and centrifuged at 40,000 x g for 20 min at 4ºC. Following centrifugation, 1 mL of the supernatant was transferred into a 1.5 mL Eppendorf microcentrifuge tube for analysis of NH_3N using Chaney and Marbach (1962) techniques but with reduced sample and reagent volumes (96-well plate reader). Another 0.5 mL of the supernatant was

filtered, diluted with 0.5 mL dH_2O, and combined with 100 µL of internal standard (86 µmol of 2 ethylbutyric acid/mL), in a 2 mL GC vial. The samples were injected into a Hewlett-Packard 6980 gas chromatograph (San Jose, CA) fitted with a custom packed column for VFA-flame-ionization detection (Yang and Varga, 1989).

Statistical Analysis

Data were analyzed with the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) as a randomized complete block design with treatment (dose level), time (expressed as h after morning feeding), and their interaction as fixed effects, and period (1 to 2) and fermenter (1 to 8) as random effects and were analyzed as repeated measures as needed using the following model:

$$Y_{ijk} = \mu + T_i + H_j + P_k + F_l + TH_{ij} + e_{ijkl}$$

Where Y_{ijk} is the dependent variable, μ the overall mean, T_i is the fixed effect of treatment, H_j is the fixed effect of time, P_k is the random effect of period, F_l is the random effect of fermenter, and TH_{ij} is the interaction between treatment and time, and e_{ijkl} the residual error assumed to be independent and normally distributed (Shapiro-Wilk test). The null hypothesis of normality was rejected ($P < 0.05$ and small Shapiro-Wilk W statistic) for molar proportion of propionate. This data was log-transformed and the transformed data was analyzed statistically. Pre-planned orthogonal contrasts were used to test linear and quadratic effects of treatment (CNSE level). Statistical significance was declared for $P \leq 0.05$ and tendencies were considered at a value of $P \leq 0.10$.

RESULTS

Diet Composition

Table 3.1 shows the ingredient and chemical content of the experiment diets. Diets for all treatments were the same, with the exception of the CNSE dose level (0, 2.5, 5.0, and 10 g/head/d). Experimental diets were formulated to meet NRC (2001) requirements for a multiparous cow (137 days in milk (DIM), 40 kg/d of milk production, and 26.0 kg/d of predicted DMI). Feed ingredients of the basal diet included: 37% corn silage, 15.0% alfalfa hay, 23.7% corn grain, and 9.5% soybean meal, and % DM was approximately 92%. Dietary OM, NDF, CP and starch content were similar between CNSE levels and averaged 93.9, 29.7, 17.0, 29.5% (DM basis), respectively. Diets fed to the fermenters were comparable to that of the donor cows.

Apparent Digestibility of Nutrients

Effects of increasing levels of CNSE (0, 2.5, 5.0, and 10.0 g/head/d) on apparent digestibility (DM, OM, NDF, and TN) are shown in Table 3.2. Under continuous culture conditions, apparent digestibility of DM (mean ± standard deviation; 57.8% ± 7.1), OM (64.4% ± 6.0), and NDF (61.2 ± 7.8) were similar ($P > 0.05$) among the treatments.

Characteristics of Fermentation

Effects of different levels of CNSE (0, 2.5, 5.0, 10.0 g/h/d) on culture VFA profile, pH, reduction potential (E*h*), and relative hydrogen score (rH) are presented in Table 3.3 Increasing the level of CNSE had no effect on total VFA concentration (72.7 ± 13.5) and molar proportion of individual VFA {(acetate (37.7 ± 2.35), propionate (27.8 ± 0.94), and butyrate (17.6 ± 2.48), isovalerate (0.96 ± 0.18), valerate (8.81 ± 0.59)} or A:P

ratio. Results indicated no differences were observed in rumen pH value (5.76 ± 0.18), E*h*, and rH between treatments. Highest pH (5.80) was observed at 10.0 g/head/d level, and the lowest pH (5.72) was observed at 2.5 g/head/d level.

DISCUSSION

Apparent Digestibility of Nutrients

Under the conditions of this study, increasing CNSE supplementation from 2.5 to 10.0 g/head/d had no effect on DM, OM, NDF, TN digestibility. These results are consistent with CNSE supplementation evaluated in vivo (Shinkai et al., 2012; Maeda et al., 2021; Goetz et al., 2021) Shinkai et al. (2012) ran 2 trials (trial 1: CNSE mixed with silica, trial 2: CNSE mixed with many ingredients); each trial was supplemented with 4 g of CNSE/100 kg of BW. In trial 1, Shinkai et al. (2012) reported a decrease in DMD from 75.6 to 72.3% but observed no effect on NDFD with the addition of CNSE. In trial 2, DMD did not differ and the digestibility of NDF tended to increase from 57.9 to 60.8% (Shinkai et al., 2012). Discrepancies between trials were attributed to pellet formation. (Shinkai et al., 2012). The authors suggest the pellet vehicle in trial 2 (CNSE mixed with alfalfa meal, defatted rice bran, silica powder, crude sugarcane molasses, and tapioca flour) might not have been as digestible as the pellet formulated in trial 1 (CNSE mixed with only silica) (Shinkai et al., 2012). In contrast, when Goetz et al. (2021) administered CNSE to transition cows at 5.0 g/head/d prepartum and 2.5 and 5.0 g/head/d postpartum they observed no effect on DM, OM, NDF, ADF, and starch digestibility. Maeda et al. (2021) reported no significant effect on DM, OM, ether extract (EE), NDF, and gross energy (GE) digestibility supplementing two doses (4 g of CNSE/100 kg of BW and 6 g

of CNSE/100 kg of BW) in beef cattle. El-Zaiat et al. (2014) reported no additive influence of CNSE on DM, OM, CP, and EE in growing lambs. Because there is little data in vitro and in vivo on the influence of CNSE on nutrient digestibility, the discussion of these effects is limited; however, the data from the current study suggests that CNSE supplementation up to 10 g/head/d would have no effect on feed digestibility in the rumen. More research is needed to validate the efficacy of CNSE on nutrient digestibility at lower and higher doses.

Characteristics of Fermentation

Cashew nut shell extract supplementation had no effect on the molar proportions of total VFA, acetate, propionate, butyrate, isovalerate, valerate, or A:P ratio in the current study. Similarly, Goetz et al. (2020) supplemented two doses (2.5 and 5.0 g/head/d of CNSE) and reported no treatment effects on rumen VFA profile in prepartum or postpartum cows. Earlier in vitro work suggested antimicrobial effects of anacardic acids (Van Nevel et al., 1971), and the results of the present study are inconsistent with these findings as well as many vitro (Watanabe et al., 2010; Oh et al., 2017) and in vivo studies (Shinkai et al., 2012; Mitsumori et al., 2014; Maeda et al., 2021). Initially, Watanabe et al. (2010) examined dose responses (0, 50, 100, and 200 μg of CNSE/mL) under semicontinuous culture conditions. At 200 μg of CNSE/mL, the authors found the highest increase in total VFA and propionate but a decrease in acetate and butyrate. Oh et al. (2017) compared the potency of monensin and CNSE in batch cultures and found that both additives changed VFA production by decreasing the molar proportions of acetate and butyrate and increasing propionate. At the suggested amount for each additive, the

extent of these alterations was lower for monensin than for CNSE (Oh et al., 2017). Moreover, these findings are supported by CNSE feeding studies; CNSE significantly increased the molar proportion of propionate and decreased the proportion of acetate (Shinkai et al., 2012; Mitsumori et al., 2014; Maeda et al., 2021). The current study found no evidence of increased propionate formation, which is one of the most important mechanisms for using metabolic hydrogen. Cashew nut shell extract levels used in this study are lower than previous supplementation levels, which may explain the study's limited impacts on fermentation profile. Overall, increasing the level of CNSE had no effect on culture pH, as pH was similar under all dietary conditions (5.76). As expected, there was a significant ($P < 0.01$) effect of pH over time (Figure 3.1). The average pH value in this experiment is not optimal for feed digestion, as cellulolytic bacteria growth and activity begin to decline below 6.0 pH (Van Soest, 1982b). Watanabe et al., 2010 observed higher pH values (6.49) in Rusitec fermenters supplemented CNSE. Lower pH could be attributed to lower-than-expected flow rate, as the pumps appeared to drift throughout the first period. To maintain similar conditions, no adjustment was made to the pumps for the second period. Although, the pH was not ideal, low pH did not appear to negatively affect nutrient digestibility and proportions of VFA. The E*h* was the lowest for the control but no effect (-208 to -249) was found between treatment levels.

CONCLUSIONS

Our results indicated that CNSE (59% anacardic acid and 18% cardol) supplemented at incremental levels (0, 2.5 5.0 10.0 g of CNSE/head/d) did not impact nutrient digestibility or fermentation under continuous culture conditions. Therefore, we can conclude that incorporating incremental levels of CNSE will not negatively affect nutrient digestibility Although, these results do not agree with previous in vitro and in vivo studies, more studies are warranted to determine the optimal supplementation dose before considering CNSE as an additive for ruminants.

Figure 3.1 Effect of time on pH with cashew nut shell extract (CNSE; 0, 2.5, 5.0, 10.0 g/head/d) supplementation.

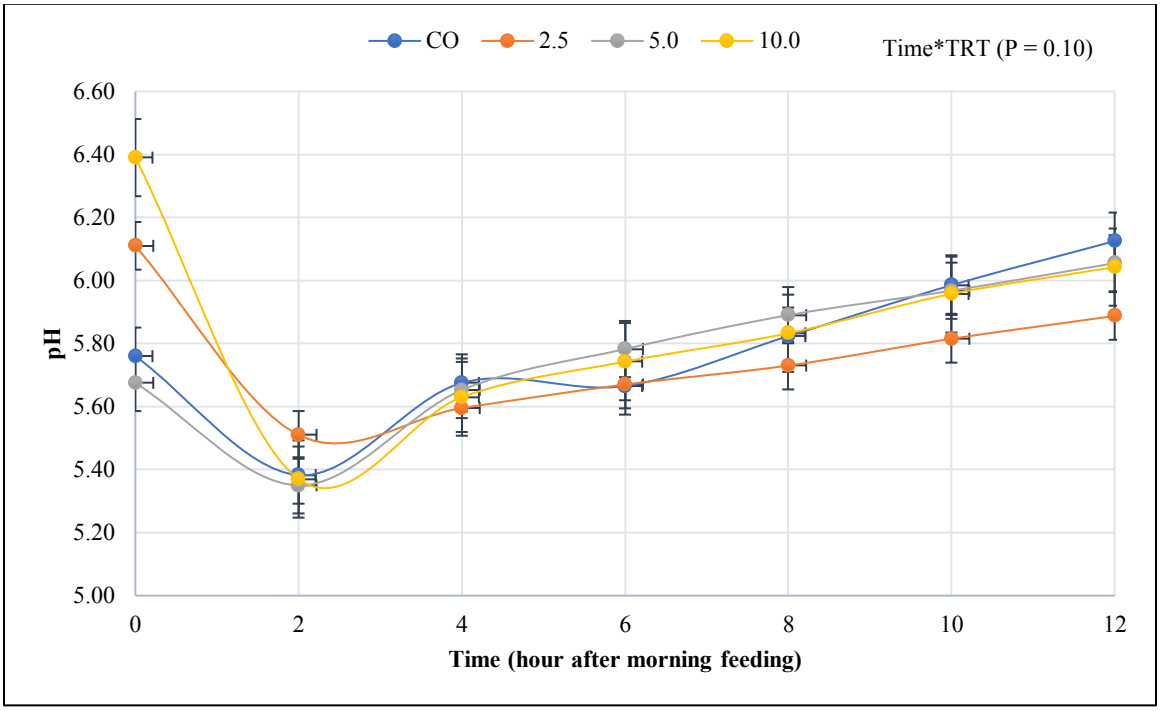

Table 3.1 Ingredient and chemical composition TMR containing different levels of cashew nut shell extract (CNSE; 0, 2.5, 5.0, 10.0 g/head/d) fed to continuous culture fermenters.

	CNSE (g/head/d)			
Ingredient[1], %	0	2.5	5.0	10.0
Corn silage	37.0	37.0	37.0	37.0
Alfalfa hay	15.0	15.0	15.0	15.0
Ground corn	18.7	18.7	18.7	18.7
Ground corn (treated)	5.00	5.00	5.00	5.00
Soybean mean (SBM)	9.50	9.50	9.50	9.50
Soyplus®	4.00	4.00	4.00	4.00
Soyhulls	8.00	8.00	8.00	8.00
Energy Booster 100	1.00	1.00	1.00	1.00
Mineral and Vitamin Mix	1.80	1.80	1.80	1.80
Chemical composition				
DM %	92.1	92.4	92.3	91.6
OM, %	94.2	93.6	94.1	93.8
CP, %	16.7	17.2	17.2	16.9
NDF, %	29.2	30.0	28.9	28.9
ADF, %	19.1	18.5	19.7	18.5
Starch, %	29.9	29.7	29.1	29.4
NFC, %	39.4	39.4	39.4	39.4
Ash, %	5.85	6.75	5.91	6.25

Table 3.2 Effect of different levels of cashew nut shell extract (CNSE; 0, 2.5, 5.0, 10.0 g/head/d) supplementation on nutrient apparent digestibility from continuous cultures.

	CNSE (g/head/day)					*P* value[1]	
Item	0	2.5	5.0	10.0	SE	L	Q
DM, %	56.3	61.2	54.4	58.4	5.24	0.97	0.91
OM, % DM	63.3	66.0	62.6	65.5	3.34	0.79	0.88
NDF, % DM	60.2	63.9	58.3	62.2	4.71	0.92	0.86
TN, % DM	38.8	43.1	30.0	34.8	5.34	0.34	0.60

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of cashew nut shell extract level supplementation.

Table 3.3 Effect of different levels of cashew nut shell extract (CNSE; 0, 2.5, 5.0, 10.0 g/head/d) on volatile fatty acids (VFA), pH, E*h*, and NH_3N concentration from continuous culture fermenters.

	CNSE (g/head/d)					*P* value[1]	
Culture fermentation	0	2.5	5.0	10.0	SE	L	Q
Total VFA, m*M*	76.8	70.5	72.3	71.2	10.9	0.46	0.51
VFA, mol/100 mol[5]							
Acetate	37.5	39.2	35.8	38.1	1.57	0.96	0.51
Propionate	27.8	26.6	28.6	28.5	8.12	0.67	0.92
Butyrate	17.8	17.6	18.4	16.7	1.81	0.61	0.57
Isovalerate	0.82	1.12	1.09	0.82	0.44	0.82	0.26
Valerate	8.70	8.71	9.30	8.52	1.56	0.93	0.58
A:P[2]	1.24	1.29	1.17	1.21	1.29	0.64	0.78
pH	5.78	5.72	5.75	5.80	0.20	0.51	0.47
E*h*[3]	-208	-212	-249	-231	21.6	0.37	0.42
rH[4]	10.9	10.6	9.41	10.0	0.78	0.13	0.17

[1]Orthogonal contrasts tested linear (L) or quadratic (Q) effects of cashew nut shell extract level supplementation.
[2]Acetate to propionate ratio
[3]Eh = Redox potential
[4]rH, Clark's exponent = ((Eh +200) / 30)) + (2 x pH)
[5]Statistical analysis was performed on log-transformed data.

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CHAPTER FOUR

OVERALL CONCLUSIONS AND FUTURE RESEARCH

This chapter summarizes the results of the experiments reported in this thesis and includes an overview of proposed future studies that will build on the findings from this work. The most significant challenge for the livestock industry is to produce more editable products to address the needs of a growing population. To combat this challenge, improvements in production efficiency are crucial. Ruminants are essential in human food production because they can convert low-quality feed to high-quality end products through the interdependent relationship they have with their microorganisms. There are inefficiencies in the rumen, but these can be improved by modifying the microbial populations, which can maximize the types of metabolic products derived from the fermentation of carbohydrates, proteins, and other nutrients. Ionophores have been successful at altering fermentation patterns; however, due to public concerns surrounding ionophores usage, researchers are looking for natural alternatives to improve rumen efficiency. As a result, CNSE's phenolic chemicals, particularly anacardic acid, have been of interest. Following the encouraging findings of Watanabe et al. (2010), in vitro and in vivo studies have been conducted to further investigate CNSE surfactant action against gram-positive bacteria and its potential as a feed additive for ruminants.

Conclusions

The main focus of this thesis was to investigate the effects of CNSE levels on nutrient digestibility and ruminal fermentation under in vitro conditions. The first study used an in vitro batch culture system to investigate the effects of increasing CNSE on

nutrient digestibility and rumen fermentation profile during the transition period (CU diet contained higher forage and FC diet contained higher starch). Under the conditions of this study, increasing CNSE levels in CU and FC diets had differential effects. For example, CNSE levels had no effect on nutrient digestibility in CU diets, but tended to linearly decrease nutrient digestibility in FC diets. Despite reductions in nutrient digestibility, there was no effect on total VFA concentration in FC diets. The molar proportions of acetate tended to decrease linearly, but the molar proportions of propionate and A:P ratio remained unchanged. When mimicking FC diets, ammonia nitrogen was lower at all levels of CNSE supplementation in a linear and quadratic manner ($P = 0.01$), which could indicate inhibition of proteolytic bacteria, increasing the amount of protein available in the duodenum. Although this study did show significant differential effects between treatment levels, the changes in rumen characteristics between the CU and FC diets indicate that the batch culture system was successful at picking up differences in dietary fermentation. The effects of CNSE on nutrient digestibility and rumen fermentation profiles were investigated further in the second study under continuous culture conditions. Nutrient digestibility and fermentation profiles were unaffected by supplementation. It's worth mentioning that the CNSE levels used in these experiments were evaluated under specific conditions. In order to better understand CNSE's impact on feed efficiency, future studies should evaluate this additive under different dietary conditions and in different stages of lactation.

Possible Future Directions

After evaluating CNSE supplementation in two different in vitro methods, the optimal supplementation dosage for dairy cows remains unclear. Before CNSE is approved as an effective feed additive, more research is needed in order to determine an effective dose, especially under in vivo conditions. So, in the future, it would be essential to continue evaluating CNSE dosages on nutrient digestibility, rumen fermentation, with an emphasis on production performance responses. Little data exists on high-producing dairy cows; therefore, in the future I would evaluate CNSE supplementation under these physiological conditions. The objective of the follow-up study would be to assess the effects of CNSE supplementation on nutrient digestibility, rumen fermentation, milk yield and composition, and the fatty acid composition of milk fat in high-producing dairy cows. Eight cannulated multiparous Holstein dairy cows would be randomly assigned to 4 doses of CNSE (0, 0.5 , 1.0, and 2.0 g/head/d) and fed a corn silage based TMR. Treatments would be administered using a 4 x 4 Latin square experimental design with four periods of 21 d each, 16 d for adaptation, and 5 d for sample collection. Feed offers and refusals will be recorded daily to calculate dry matter intake (DMI). Milk yield would be measured during milking (morning and evening) the last 3 d of each period. Milk samples would be collected on d 17 and 18 for the analysis of fat, protein, lactose total solids, and somatic cell count. Additionally, fecal samples would be collected to determine the total tract apparent digestibility of nutrients (DM, OM, NDF, TN, starch). Over the last 24 h of each period, rumen contents would be sampled every 4 h and stored until VFA and NH_3N analysis. This potential study could help us better understand the

effects of CNSE supplementation in dairy cows because feeding directly to the animal is the most accurate way to determine nutrient digestibility, fermentation and production performance.

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