EFFECTS OF PROTEIN CONCENTRATION AND BETA-ADRENERGIC AGONISTS ON RUMINAL MICROBIAL COMMUNITIES IN FINISHING BEEF HEIFERS

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Thank you to my husband, who has supported and encouraged me to keep follow my dreams. I am glad to have you in these crazy adventures around the country and, the most important, to have you in my life forever. I am willing to keep proving my love to our growing family. I am very grateful for our future projects, for our rainbow baby Julia, for our cats Sofia and Jersey, and for all the good things and adventures that are coming for us.

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ABSTRACT

To improve animal performance and modify growth by increasing lean tissue accretion, beef cattle production has relied on use of growth promoting technologies such as beta-adrenergic agonists. These synthetic catecholamines, combined with the variable inclusion of rumen degradable (RDP) and undegradable protein (RUP), may improve feed efficiency and rate of gain in finishing beef cattle. However, research about the impact of beta-adrenergic agonists and protein level and source on the ruminal microbiome is limited. The objective of this study was to determine the effect of different protein concentrations and beta-adrenergic agonist (ractopamine hydrochloride; RAC usage) on ruminal bacterial communities in finishing beef heifers. Heifers (*n*=140) were ranked according to body weight and assigned to pens in a randomized complete design to 6 treatments, containing 3 protein treatments (Control: 13.9% CP, 8.8% RDP, and 5.0% RUP; High RDP: 20.9% CP, 13.4% RDP, 6.1% RUP; or High RUP: 20.9% CP, 9.1% RDP, 10.4% RUP) and 2 RAC treatments (0 and 400 mg/day). Rumen fluid samples were collected from heifers by oral lavage 7 days before harvest. The DNA from the samples were sequenced to identify bacteria based on the V1-V3 hypervariable regions of the 16S rRNA gene using the Illumina MiSeq. Sequences and data from the treatments were analyzed using the R environment and PROC MIXED in SAS 9.4 (SAS Inst.; Cary, NC). Beta diversity was analyzed using PERMANOVA based on PCoA Bray-Curtis distances and were significant among the protein and RAC treatments ($P < 0.05$). Alpha diversity metrics such as Chao1 and Shannon diversity indices were not significantly different ($P > 0.05$). Differences among treatments at variable taxonomic levels after analyses through DESeq2 were

significantly different for the main effects of protein concentration, rather than the interaction of protein and RAC treatments ($P < 0.05$). These results suggest possible effects on the microbial communities with different concentration of protein, but limited positive impact with RAC. However, both may potentially act synergistically to improve performance in finishing beef cattle.

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CHAPTER ONE
LITERATURE REVIEW

Introduction

Ruminants have the capability to utilize different resources or foodstuffs and transform them by different process into high quality protein for human consumption. To meet growing protein demand for humans, beef producers must implement technologies to grow beef cattle efficiently, while considering and managing animal welfare (Duffy, 2019).

The United States livestock inventory has undergone several changes in the past 50 years. After reaching its highest numbers in 1985, beef cattle numbers progressively declined until reaching a low in 2014. However, the beef cattle inventory gradually increased again in 2018, reaching approximately 33 million head (USDA, 2018). Currently, the inventory is slightly below the previous year (2020; 93.8 million head) with a total of 93.6 million head (USDA, 2021). The beef industry faces a decline of cattle numbers in United States, forcing producers to yield more pounds of meat with fewer animals. An objective of beef cattle production is to increase muscle mass, which can be accomplished through improvements in feed efficiency. For this reason, the components and nutrient composition of finishing cattle diets have historically been altered by increasing the use of byproducts from the corn milling industry. These byproducts result in diets with an excess supply of dietary protein (Samuelson K. L., 2016). Research has shown limited enhancements in animal performance when the crude protein (CP) concentration in finishing diets was greater than 13% (Galyean, 1996, Gleghorn et al., 2004). However, if CP was greater than 15.5% [by the combination of protein degradability], results demonstrated a possible metabolic

cost related with ammonia detoxification from the liver. These results indicate that the protein degradability likely performs a critical role in animal performance (Lobley et al., 1995).

With continuing interests regarding the improvement of lean mass yield, producers and researchers continue to develop new or improved technologies that modify and improve the management and performance of finishing cattle. Technologies that improve performance of the animal include growth-promoting steroidal implants and beta-adrenergic agonists, which are generally fed to beef cattle between the last 70 to 120 days before harvest to increase muscle growth (Nichols et al., 2005). Utilization of implants in the beef industry has been common in the US since 1956 with the approval of diethylstilbestrol (DES), and since then, many varieties of implants have been developed with the purpose of maximizing productivity and minimizing negative costs to meat quality. By recognition of their value in human medicine, beta-adrenergic agonists and their respective host-receptors, started to be deeply studied in the 1970s, showing their work as a repartitioning agent, redirecting energy to protein accretion and reducing lipid deposition (Harsh, 2018). The economic benefits of the beta-adrenergic agonists made them an excellent tool for feedlot consulting nutritionists, who reported that 85% of their customers used beta-adrenergic agonists during the finishing period (Samuelson K. L., 2016)

In 2003, ractopamine hydrochloride (Sanchez et al.), a beta-adrenergic agonist (synthetic catecholamine) marketed under the commercial trade name Optaflexx (Elanco Animal Health. Greenfield IN), was approved for use in cattle feed in the United States at a rate of 70-430 mg/animal/day during the last 28 to 42 days prior to harvest. (Avendaño-Reyes et al., 2006; Arp et al., 2014). Years later, zilpaterol hydrochloride (Merck Animal Health, Summit, NJ) was accepted for the use in cattle feed in the United States at a proportion of 8.3 mg/kg on a dry matter

basis in a complete feed for the last 20 to 40 days prior to harvest. However, in contrast with ractopamine hydrochloride which has a zero-day withdrawal, zilpaterol hydrochloride required a withdrawal period of 3 days to ensure animals without drug residues. Ractopamine hydrochloride has been used in cattle to gain muscle mass, showing greater results when it is included in the diet, in parameters such a final body weight (BW), carcass weight, and animal performance. In finishing steers, the dietary supplementation of RAC resulted in 7.3 to 10.1 kg greater final BW (Gruber et al., 2007, Bryant et al., 2010), whereas in finishing heifers, animals were 8.3 to 10.6 kg greater in final BW. RAC also improved the average daily gain (ADG), resulting 16 to 32% greater ADG compared to animals without the supplementation of RAC (Avendaño-Reyes et al., 2006, Abney et al., 2007, Bohrer et al., 2014).

The interaction between the RAC and dietary protein has been investigated. Studies have demonstrated that animals fed diets high in ruminally degradable protein in combination with RAC, improved the response of RAC (Walker et al., 2006; Beermann et al., 1986). Given this information, researchers have also observed the interaction of RAC with ruminal microbes. Naturally occurring catecholamines have been shown to affect certain types of microorganisms; including bacteria. Walker et al., (2006) suggested that supplementation with RAC could alter proteolysis processes in the rumen, affecting the availability of the rumen microbiota to utilize ruminally degradable protein. The group also observed that the ratio between protein degradable fractions (RDP and RUP) could influence the response of RAC. Therefore, it is important to explore how different protein concentrations in combination with beta-adrenergic agonists could impact the ruminal communities of beef cattle and, evaluate how the interaction between beta-adrenergic agonists and dietary protein could improve animal performance and efficiency.

Beta adrenergic agonists overview

Beta-adrenergic agonists

To accelerate animal growth by improving lean tissue accretion and address the global demand for additional beef, livestock production has commonly used beta-adrenergic agonists. Beta-adrenergic agonists (β-AA) are phenethanolamine compounds with similar physical and pharmacological characteristics to endogenous catecholamines, such as epinephrine and norepinephrine (Bell et al., 1998). The β-AA have been shown to improve feed efficiency, increase the rate of gain, and decrease deposition of fat in the carcass through stimulation of adrenoreceptors situated on the membrane into muscle and adipose tissue (Vasconcelos et al., 2008, Elam et al., 2009, Montgomery et al., 2009). The structure of all β-AA conforms to a six-membered aromatic ring, a hydroxy group bound with a β carbon, a charged N in the ethylamine side chain, and an adjacent R group to the aliphatic N, which is required for biological activity (Smith, 1998). However, some differences in the substitution of the aromatic and the R group, which are essential for subsequent activity, can contribute in the affectation of the tissue longevity, metabolism and the affinity of the receptors (Smith, 1998), while help to prevent a rapid deactivation of the β-AA (Smith, 1998). Moreover, the presence of the aliphatic amino group is important for the correct function of the β-AA. The alkaline pKa that these amino groups have permits them to exist in a protonated state in different tissues at physiological pH and be ionized at the beta-adrenergic receptors (Smith, 1998).

Adrenergic receptors (α and β-AR) are members of a complex family of G protein-coupled receptors (GPCR). Adrenergic receptors are positioned in the plasma membrane in mammalian cells, which contain seven hydrophobic membrane-spanning regions with three internal and

external segments associated with the N-terminus and C-terminus (Mills et al., 1995, Johnson et al., 2014). The C-terminus has a function of regulatory phosphorylation, but is inactivated by the phosphorylation at the ring 3 located in the G proteins (Johnson et al., 2014). The β-AA bind to those β-AR to produce the activation of the G protein. The activation stimulates the α subunit of the G protein to dissociate from the γ and β subunits and activates Adenylyl Cyclase enzyme by the binding of GTP. The reaction produces cyclic Adenosine Monophosphate (cAMP) which is recognized as one of the main intracellular signaling molecules. The mechanism of action of cAMP requires binding with Protein Kinase A (PKA) to phosphorylate intracellular proteins, such as, hormone sensitive lipase, an enzyme for adipocyte triacylglycerol degradation (Mersmann, 1998) (**Figure 1.1**).

The phosphorylation helps to increase the transcriptional action of the cAMP response element binding protein (CREB), which is phosphorylated prior by action of PKA. The transcriptional activity of CREB provides the tools for β-AR agonists to facilitate the transcription of a number of genes in the mammalian cells. However, enzymes, such as acetyl-CoA carboxylase, and long-chain fatty acid biosynthesis enzymes can be inactivated by the phosphorylation (Mersmann, 1998). The β-Adrenergic receptors have been historically classified into different subtypes, represented as β1, β2 and β3 (Bylund, 1994). Research has demonstrated the difficulty in classifying theses receptors due to the selectivity, mechanisms for signal transduction, differences in ligand binding affinity, physiological effects of these receptors, and the different distribution across species and tissues (Bylund, 1994, Mersmann, 1998). Studies have shown the quantification and the distribution of the β-AR in human tissues (80 β1: 20 β2- AR ratios) and rat tissues (15 β1: 85 β2- AR ratios) (Minneman et al., 1979, Bristow et al., 1986).

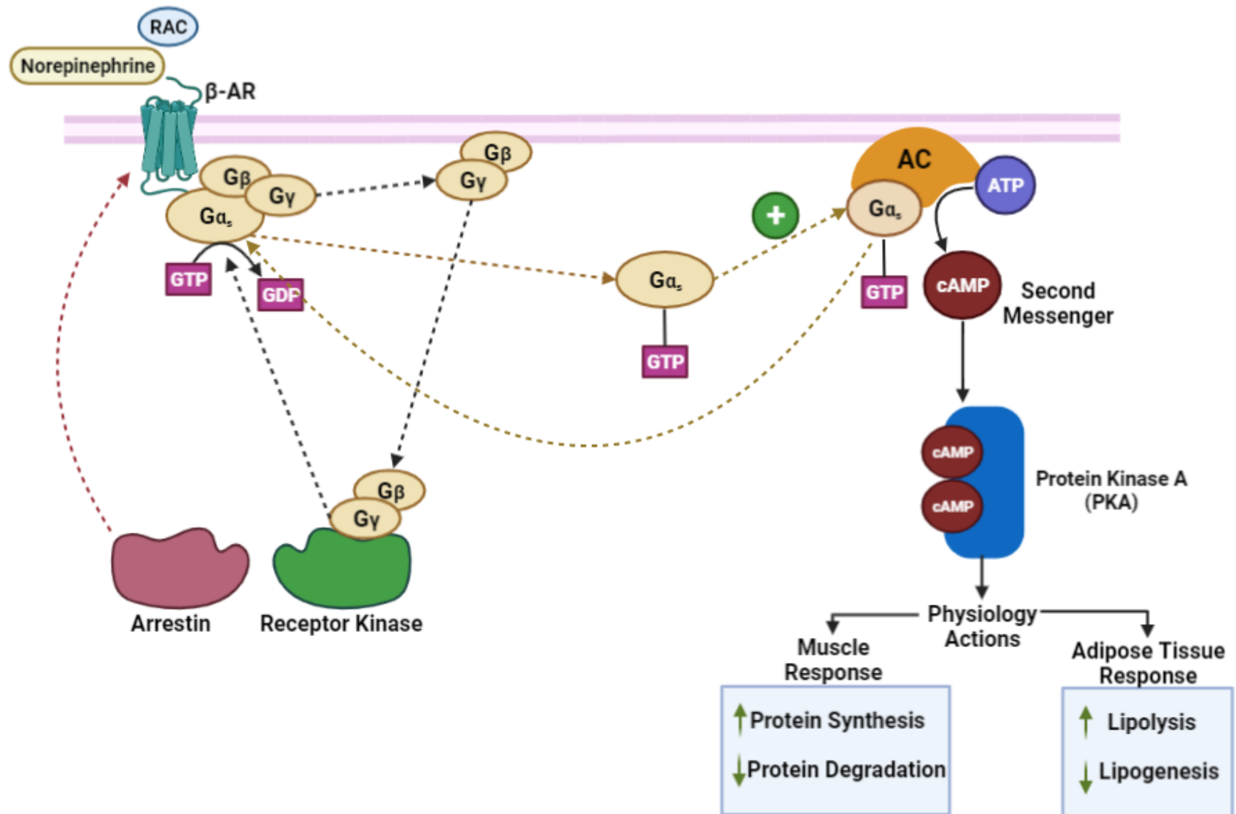


Figure 1.1. Mechanism of action of beta-adrenoreceptors. (RAC) ractopamine hydrochloride, (β-AR) beta-adrenergic receptor, (G_α, G_β, G_γ) Gs protein, (AC) Adenylyl Cyclase enzyme, (ATP) Adenosine Triphosphate (cAMP) Cyclic Adenosine 3',5'- Monophosphate. (Adapted from Bach et al., 2005 and Johnson et al., 2014)

Also, the abundance of β-AR subtype mRNA transcripts in different tissues of porcine was observed by Liang and Mills (2002), indicating greater ratios of β1 over β2 in subcutaneous adipose tissue (81:19), skeletal muscle (59:41), heart tissue (72:28) and lung tissue (58:42). Despite the study of the characterization of β-AR subtypes in different species, such as humans (Krief et al., 1993) and porcine (Mersmann, 1998, Liang and Mills, 2002), information regarding β-AR in bovine tissues is still relatively limited. Some reports about the proportions of the β-AR subtypes in bovines have shown that more than 99% of β2-AR are located in the skeletal muscle and around 90% in the adipose tissue (Sillence and Matthews, 1994). Ricks et al. (1984) postulated that by the stimulation of hydrolysis or lipolysis and the inhibition of *de novo* fatty acid biosynthesis, β-AA can decrease the adipose tissue accretion, and contrarily produce the increase of muscle mass by the inhibition of protein turnover, encouraging myofibrillar protein synthesis (**Figure 1.2**).

One of the important characteristics of β-AA is the rapid absorption after an oral administration (Harsh, 2018). Ungemach (2004) observed that after dosing β-AA, concentrations peaked in the plasma after 0.5 – 2 hours, and total elimination was observed 6 to 7 hours after the initial administration of β-AA in rats, dogs and swine. Extensive research exists regarding the rate and degree of absorption of β-AA in cattle and other species (Smith and Paulson, 1997). Some studies affirmed that β-AA are absorbed rapidly in the gastrointestinal tract through passive diffusion, due to the neutrality of the pH which prevents the formation of cations at the phenethanolamine nitrogen and helps the absorption through the intestinal mucosa (Smith, 1998, Ungemach, 2004). However, the information about the site of absorption in ruminant species is still limited.

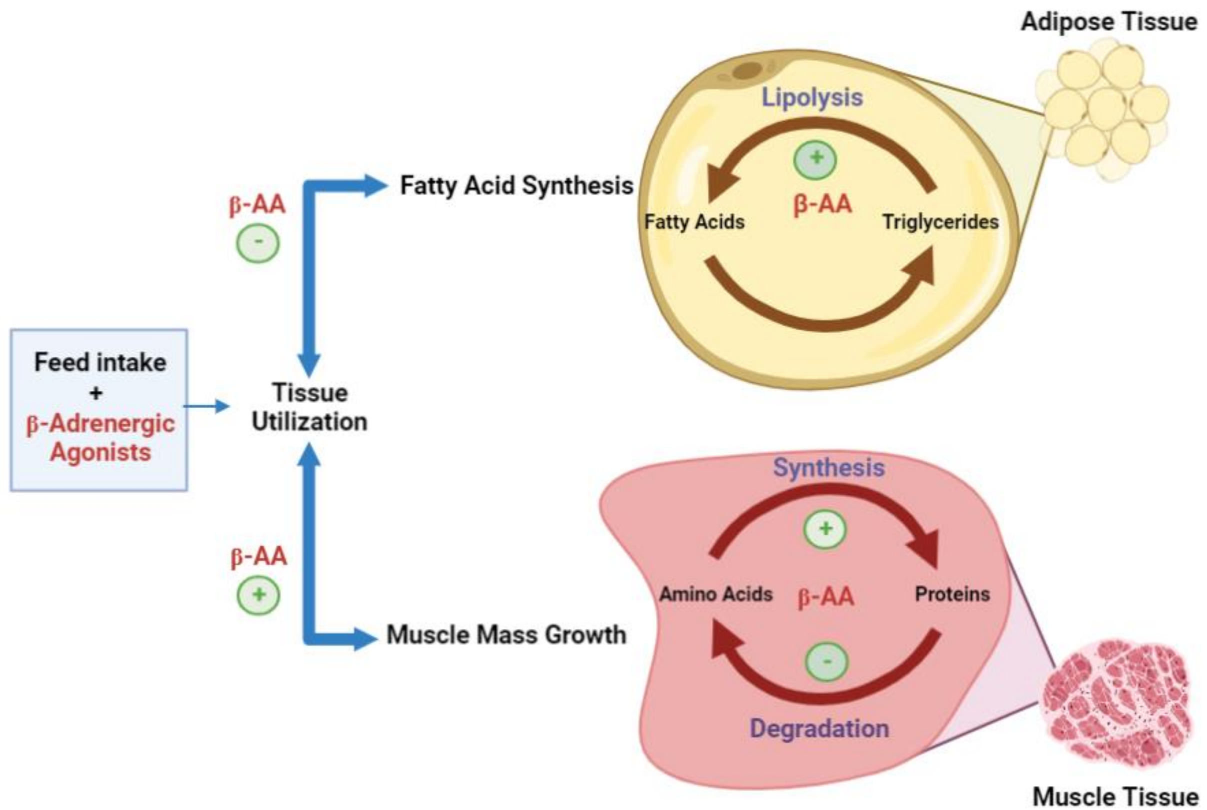


Figure 1.2. Proposed mode of action of beta-adrenergic agonists in the accretion of muscle growth and the adiposity reduction (adapted from Johnson et al., 2014).

Mechanism of action

Effects on skeletal muscle deposition

The supplementation of β-AA impacts growth by increasing the accretion of skeletal muscle via muscle hypertrophy and directly reducing lipolysis (NRC, 1994). The accretion of skeletal muscle results in greater synthesis of protein and/or lower degradation of protein, improving animal muscularity (Mersmann, 1998, Laudert et al., 2007). β-AA benefits can be short-term; different studies have shown the desensitization on β-AR in experiments with rats and pigs, where rats gained weight the first 7 days, then the response of the β-AA decreased to zero by day 14. A similar pattern was shown with pigs, where the response of β-AA was positive during the first week, but the response declined to zero by week 7 (Williams et al., 1994). These reactions created a hypothesis that the down-regulation of the β-AR in the adipose tissue could prevent the complete expression of the receptors, generating no changes in the rate of adipose tissue accretion (Mills, 2002). For this reason, β-AA are used in the final finishing phase of feeding in animal production (Mills, 2002).

The hypertrophy in animals fed with β-AA results in greater blood flow into the muscle, allowing greater flow of nutrients and improving the efficiency of the muscle cell growth (Mersmann, 1998, Rathmann et al., 2009). In addition to muscle hypertrophy, extensive studies have demonstrated that β-AA administration can also increase the fiber diameter of the muscle, as well as affect the different muscle fiber types (myosin heavy chain (MHC) type II to MHC type I). According with NRC (1994), the β-AA action increases the growth of type II fibers, contrasted with the results on type I fibers. However, Gonzalez et al. (2007) noted that during supplementation with ractopamine hydrochloride (RAC) in cull beef cows, the fiber type I had a diameter increase, but could not

detect a response on fiber type II. The increase of muscle growth, and improvements in other characteristics, also have a negative consequence, due to reduced marbling scores and elevated beef toughness, producing an increase of glycolytic fiber types (Rathmann et al., 2009, Baxa et al., 2010). It has been proposed that insulin growth factor-I (IGF-I) could be implicated in skeletal muscle hypertrophy with the presence of β-AA, due to decreased degradation of protein and increased protein synthesis (Johnson et al., 2014). An experiment with lambs fed 10 ppm of cimaterol (β-AA), resulted in a reduction of the IGF-I levels by 46.5% at day 42 and 21.5% at day 84 in comparison with control animals (Beermann et al., 1987). In Holstein animals supplemented with RAC, Walker et al. (2006) observed an increase of longissimus muscle (LM) IGF-I mRNA concentrations compared to concentrations observed with animals in the control. Additionally, IGF-I is known to stimulate the division of muscle satellite cells by mitosis, which assist postnatal muscle hypertrophy. Therefore, it is necessary for further studies to determine the activity of IGF-I in satellite cell proliferation during the stimulation of β-AR by the β-AA in skeletal muscle hypertrophy (Johnson et al., 2014).

Effects on protein accretion

The synthesis of protein has been observed to increase in porcine skeletal muscle by the supplementation of RAC (Bergen et al., 1989). These studies indicate that the use of RAC can induce muscle protein accretion, enhance protein synthesis, and inhibit protein degradation (Johnson et al., 2014). However, protein accretion is recognized as an unproductive, although essential, process which is responsible for approximately 20% of total outflow energy in livestock species during growth (Reeds et al., 1985). The accretion of protein is divided in two different process, catabolic and anabolic processes, and β-AA have been involved in these processes by way

of different pathways (Harsh, 2018). Due to the presence of β-AA in those catabolic and anabolic protein processes, their effects have demonstrated increases in the activity of the proteins involved in the degradation by the calpain system (Harsh, 2018). The activity of CREB activation also assists the reduction of protein degradation by the increase of calpastatin production, directly inhibiting calpain proteases (Cong et al., 1998).

The activation of the targets affecting protein synthesis is a result of the activation of protein kinase B (Akt) signaling. One of the targets activated is the mammalian target rapamycin (mTOR), which increases protein synthesis by the activation of the ribosomal protein s6 kinase ($p70^{s6k}$) (Harsh, 2018). Ribosomal protein s6 kinase is correlated with elongation, translation, and indirectly implicated in the activation of an eukaryotic translation initiation factor (eIF4E), which has an important role in the initiation of protein translation (Norton and Layman, 2006). Another important function of Akt is the inhibition of the protein breakdown process by phosphorylation and inactivation of forkhead box O, which is a transcription factor necessary for E3 ubiquitin ligases (Sandri et al., 2004, Stitt et al., 2004). Other mechanisms involving the activation of the Akt include the β- arrestin, phosphatidylinositol 3- kinase (PI3K) and the initiation of cAMP response element binding protein (CREB) (Berdeaux and Stewart, 2012).(**Figure 1.3**)

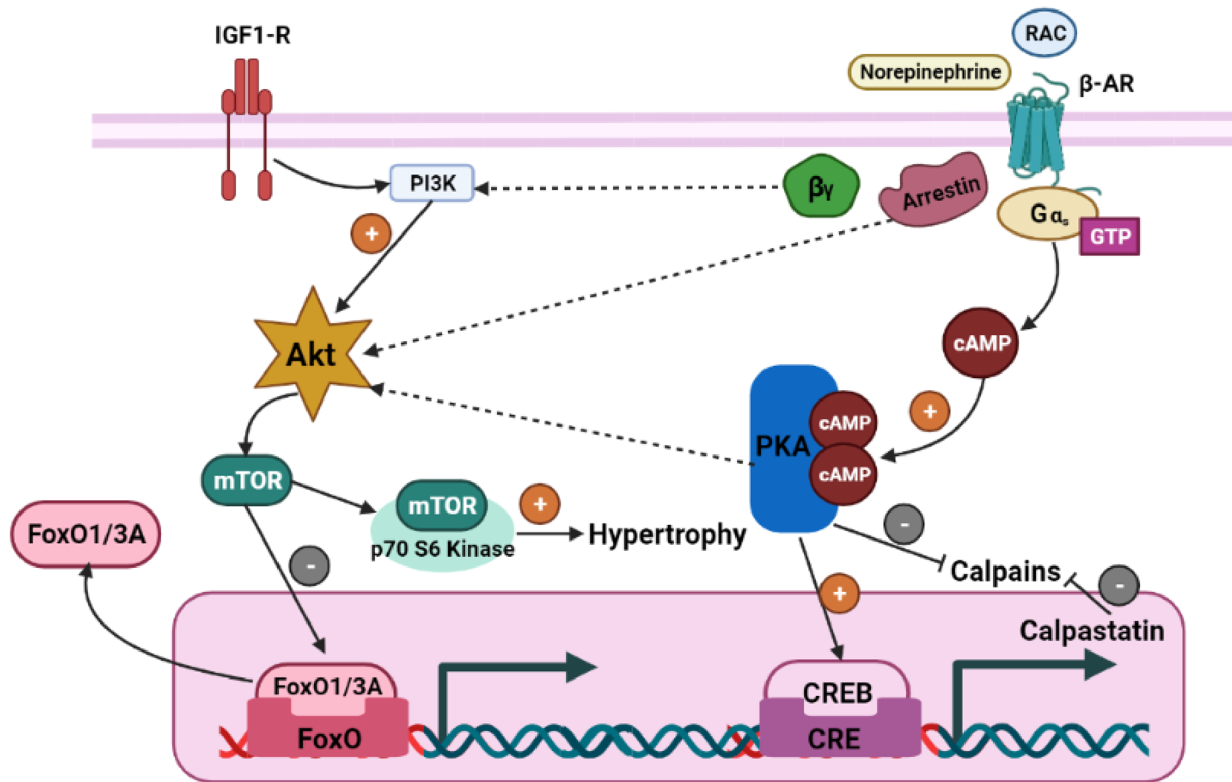
Pringle et al. (1993) observed that lambs fed with β-AA, CREB and Akt increased calpastatin production and reduced the activity of calpain. These results were supported by other researchers with similar results of greater calpastatin in steers supplemented with β-AA (Killefer and Koohmaraie, 1994, Strydom et al., 2009). Regardless of the positive effects of β-AA signaling on the target activity, is important to understand that the principal effect in protein accretion is directed by the increase of muscle hypertrophy, without affecting the myonuclei. Likewise various studies

reported cell proliferation increased, however, the fusion of the satellite cells did not show the same response (Grant et al., 1990).

Effects of catecholamines and beta-adrenergic agonists on the microbiome

Rumen microbes- bacteria

The rumen is a continuous fermentative ecosystem that provides an ideal anaerobic environment to maintain the variable populations of microorganisms (Cobellis et al., 2016). The microbial community in the rumen is divided into three domains of life: Bacteria, Archaea, and Eukarya. These types of microbes include bacteria, archaea, and two groups of eukaryotes of protozoa and fungi. The communities of bacteria and protozoa are the largest groups with regard to microbial biomass, representing 90% of the total microbial biomass (Weimer, 2015). The rumen microbial populations interact through various biotic relationships such as mutualism (benefits for both microorganisms), and commensalism (benefits for one without influencing the other), allowing the ruminant to obtain the essential nutrients for its nutrition through microbial fermentation processes (Baldwin and Allison, 1983). Yokoyama and Johnson (1988) considered that the best example of a cooperative symbiotic system with regard to animal-microbial symbioses is that of the rumen, where microbes have been represented as endosymbionts in the course of the evolution. Microbial communities in the rumen are traditionally characterized by their physiological, morphological, and ecological differences among them, however, the majority have the capacity to breakdown, ferment, and/or store polysaccharides and proteins derived from plants (Firkins and Yu, 2006). The microbiota in the rumen provide stability in the rumen ecosystem through their resistance, resilience and functional redundancy.



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This stability increases the ability of adaptation and acclimation in terms of dietary changes or management approaches (Edwards et al., 2008, Weimer, 2015). The ability to degrade plant substrates is due to the metabolic activity of bacteria species, protozoa and fungi (Restrepo and Suárez, 2005). Several authors have classified rumen bacteria populations grouping them according to their morphology, the substrate they ferment, the final product generated in fermentation, or interactions with food particles. Wang et al. (2017) supported McAllister et al. (1994) and classified bacteria into five subgroups based on the interaction with food particles: 1) free – bacteria carried in the rumen liquid phase, 2) bacteria weakly related with feed particles, 3) bacteria firmly attached to feed particles, 4) bacteria connected with rumen epithelium, and 5) bacteria adhered to the surface of protozoa or fungal sporangia.

The substrate the microbes ferment or the final fermentation products in the rumen are the most practical to understand the degradation of the food ingested by the ruminant and to elucidate metabolic routes and fates (Yokoyama and Johnson, 1988). Ruminal bacteria are commonly recognized based on the use of cellulose, hemicellulose, sugars, intermediate fatty acids, proteins, and lipids, as well as methane production, peptide use, and ammonium production. Within the ruminal microbial network, overlaps and redundancies exist among species of bacteria because most are capable of fermenting various substrates (Yokoyama and Johnson, 1988).

Peptide fermentation is due to the action of bacteria such as *Bacteroides amylophilus*, *Bacteroides ruminicola*, *Butyrivibrio fibrisolvens* and *Streptococcus bovis* (Cobarrubias et al., 1996). Research supports that up to 38% of the total of ruminal bacteria are proteolytic and indicates the existence of at least three microbial proteases: cystine- protease, serine- protease and metallo- proteinase (Yokoyama and Johnson, 1988, Van Soest, 1994). The ammonia-producing bacteria, such as

Prevotella ruminicola, *Bacteroides ruminicola*, *Megasphaera elsdenii*, *Selenomona ruminantium* and *Butyrivibrio sp.*, obtain ammonia from urea hydrolysis and protein deamination. These group of bacteria represent 5% of the total population of the rumen. *Prevotella ruminicola* is one of the most important bacteria in this group, because it is the largest ammonium producer in the rumen (Van Soest, 1994). Maximum levels of ammonia in the rumen are reached two hours after feed ingestion, coinciding with the maximum growth of proteolytic bacteria (Galindo et al., 1993). Bacterial protein is synthesized by total or partial degradation of crude proteins, amino acids, or NH_3 from the diet (Carulla et al., 1998). Microbial crude protein (MCP), ruminal undegradable protein and endogenous crude protein play a part into the passage of metabolizable protein to the small intestine to be absorbed and utilized by the animal (NRC, 2001).

The solubility of the protein in the diet, and characteristics of the protein, such as the structure, animal intake, and feedstuff size, are some reasons why proteolysis in the rumen can be variable (Walker and Drouillard, 2012). The proteolytic activity of microorganisms in the rumen is around 75%, as these enzymes interact in different digesta fractions of the rumen (Brock et al., 1982). Bacterial species in the rumen are interconnected, and the competition and the selection of nutrients is crucial for the microorganism’s survival. For this reason, changes in the environment of the rumen can affect the entire population of the ruminal microbiome (Walker and Drouillard, 2012)

Protein degradation in the rumen.

In agricultural production, the use of feed nitrogen (principally in the form of protein) in animal diets had been inefficient. The primarily concern with feed nitrogen (N) is the excretion of ammonia, which is the base of distinct environment consequences. For example, ammonia acts as

a substrate for nitrification to nitrate and an air pollutant, negatively impacting animal and human health, and producing irreversible ecosystem damages (Weimer, 2015). For optimal productivity, nutritionists have the responsibility of creating alternatives to waste N disposal, improving protein efficiency and use of N, and aiming to reduce feed costs per unit of lean tissue and the implementation of other nutrients in the diet that will enhance production (NRC, 2001).

In finishing cattle diets, ingredients commonly utilized are byproducts of corn millings, including corn gluten feeds and distiller's grains, because they can include over 30% CP. The proportion of those byproducts in diets of finishing cattle is greater than 50% to substitute high energy feedstuffs (Vasconcelos and Galyean, 2007). Another compound typical of finishing cattle diets is urea; a large source of non-protein N (NPN) that is offered in diets as an alternative source of rumen degradable protein. Urea can be transformed in the rumen by the ruminal microorganisms into microbial CP (Bach et al., 2005). It is important to understand the precautions before the use of the combination of byproducts and urea, due to grain byproducts contain an excess in CP (over 30%) and in combination with NPN sources may increase the concentration of dietary crude protein, exceeding the requirements for crude protein (NRC, 1996).

Dietary protein is commonly denoted as a crude protein (CP). The requirements of CP for finishing cattle are between 12.5% to 13% of dry matter (Duff, 2007). However, recent studies have suggested greater levels of protein (13.5%) in the diet for finishing cattle, with the use of nitrogen sources such cottonseed meal, soybean meal, grain coproducts, and urea (Vasconcelos and Galyean, 2007). In general, all the protein contained as components of animal feed are recognized to have a certain "pass-through" effect percentage. A greater percentage of these are degradable in the rumen, and therefore, a lower percentage are usable (digested and absorbed) directly in the

small intestine. Highly degradable proteins in the rumen can be converted into NH_3 (N), regardless of their quality. Ammonia is an important substrate for ruminal bacterial protein production, which then pass into the intestine as a natural, high-quality pass protein source to be digested and absorbed by the animal (León and Chicco, 1991).

Crude protein in ruminants is divided in two important components of protein, rumen-degradable protein (RDP) and rumen-undegradable protein (RUP). These types of protein have separate functions. RDP offers a combination of peptides, free amino acids, and ammonia, which is important for microbial growth and the synthesis of microbial crude protein (MCP). This MCP provides the majority of amino acids that pass to the small intestine. RUP is the secondary source of absorbable amino acids to the animal (NRC, 2001). When the animal consumes RDP, it is degraded by the microbes in the rumen, whereas RUP will escape the reticulorumen, move directly to the abomasum and the small intestine for post-ruminal digestion, and be absorbed by the animal (Bach et al., 2005). With regard to RDP, the microbial community can start the absorption of feed particles and begin the breakdown of the peptide bonds of CP by the use of proteases (Russell, 2002). By degrading CP into peptides or free amino acids; this end product of degradation will be introduced to the microbe. Further, if energy is available, these substrates will be used for the synthesis of MCP (Bach et al., 2005). Nevertheless, if the energy required for this process is not available or RDP is provided in greater proportions than the rumen microbial capacity for microbial CP synthesis, the excess protein and amino acids will be deaminated, fermented, producing volatile fatty acids (VFA; such as acetate, propionate and butyrate) and ammonia (**Figure 1.4**). Microbial ammonia is then expelled, released into the rumen for absorption through

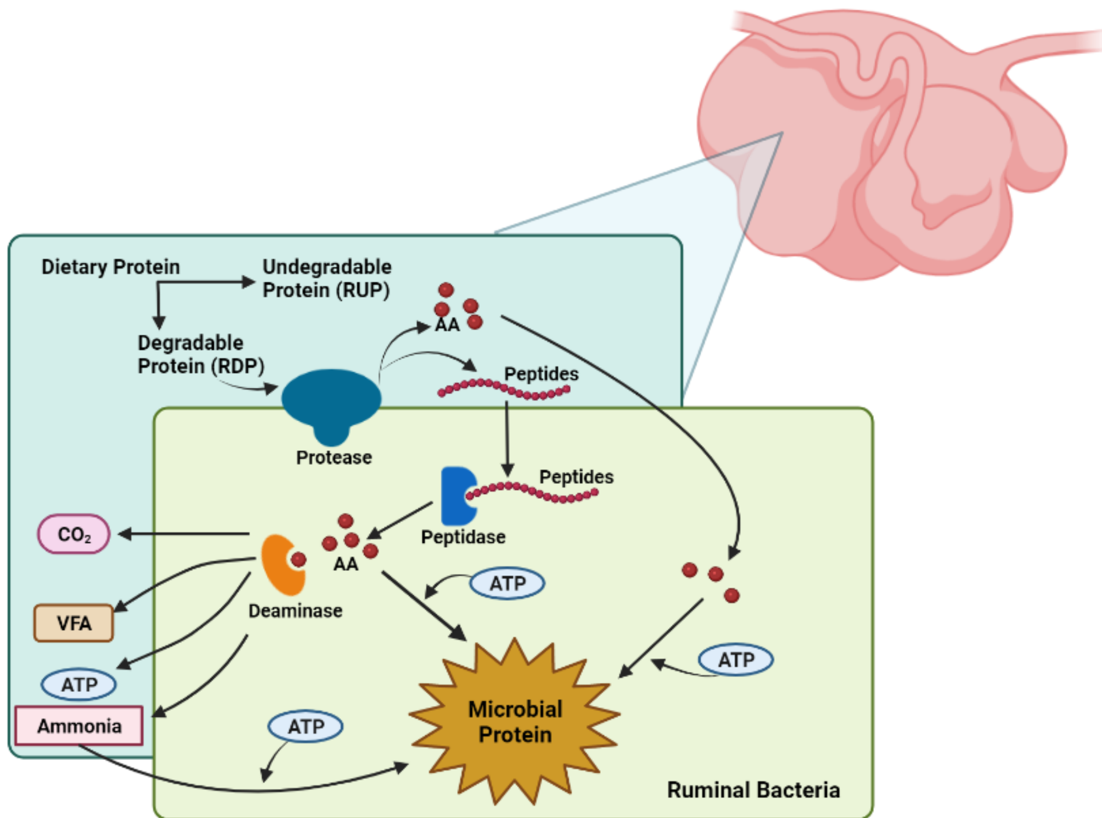

Figure 1.4. Schematic representation of protein degradation process in the rumen. (Adapted from Bach et al., 2005).

the rumen wall and further into the bloodstream, in the liver the overflow of ammonia is converted to urea and excreted in the urine (Church, 1993, Bach et al., 2005).

Conversely, when the concentration of ruminal ammonia is low, urea is recycled by two different pathways: via the rumen wall or via the saliva, with the purpose of providing an extra source of N when the dietary protein is limited (Church, 1993). The accumulation of amino acid intake may be a limiting factor of protein degradation, suggesting that the manipulation of protein degradation can be complete by the proteolysis modulation and by some modifications in peptidolysis and deamination (Bach et al., 2005).

Microbial protein synthesis

The protein produced by ruminal microbes represents the main source of amino acids and approximately 85% of the total absorbable protein (NRC, 2001) in the ruminant diet. The availability of MCP in the rumen depends on the characteristics of the nutrients in the diet, such as amount of carbohydrates and proteins, and the nutrient-use efficiency of the microorganisms. The synthesis of MCP use and require ATP for maintenance and growth, and requires peptides, amino acids, and ammonia to be used in MCP (NRC, 2001). Depending on the amount of RUP in the diet of beef cattle, MCP can represent approximately 50% of the metabolizable protein (NRC, 1996). Feedstuffs play an indispensable role in microbial protein synthesis because synthesis decreases in animals fed high-concentrate diets, due to the lower pH in the rumen (NRC, 1996); and also decreases with low quality forages, due to slow degradation of carbohydrates.

Higher levels of Non-structural carbohydrates decrease the concentration of ammonia in rumen, stimulating microbial protein synthesis (Lewis and McDonald, 1958) and the utilization of the nitrogen by the microbes is more efficient. Stern et al. (1978) observed that by increasing the

dietary inclusion of pectin, cellulose, and hemicellulose, concentrations of ammonia increased, and as a result, microbial protein synthesis decreased. Importantly, microbes in the rumen are specialized with regard to their fermentation of structural carbohydrates and non-structural carbohydrates, utilization of ammonia, and fermentation of amino acids and peptides as a primary source of nitrogen (Russell et al., 1992). To improve the rumen environment and growth of bacterial species, dietary alternatives can be recommended regarding a mixture of dietary components (e.g. forages and concentrates), which can also increases microbial protein synthesis (Pathak, 2008).

Ammonia, as a principal source of nitrogen for ruminal bacteria growth, can affect protein degradation in the rumen, and directly affect negatively the synthesis of MCP, due to a lower supply of ATP from higher degradable protein in the rumen compared to digestible carbohydrates (Firkins, 1996). However, it is important to prevent free ammonia in the blood because it can potentially result in toxicity and could be dangerous to the animal (Samuelson K. L., 2016). To improve and stabilize growth rates of bacteria in the rumen, Satter and Slyter (1974) recommended a concentration of 50 mg of ammonia N/L as an acceptable amount for ruminal bacteria. As a response of greater concentrations of ammonia produced by excess RDP or excess amino acids supplied by MCP, detoxification of ammonia through ureagenesis can occur (Lobley et al., 1995). However, ammonia detoxification may impact energy metabolism, due to increased energy cost of the process, resulting in negative implications on animal performance (Lobley et al., 1995). Imbalances in the energy: protein ratio cause serious effects on animal health and performance and, reproductive efficiency. Issues such as degradation of the ruminal epithelium, hepatic toxicity,

ketosis, pneumonia, mastitis, and laminitis, are frequently associated with imbalance in energy: protein ratio (Giraldo et al., 2005).

Effects of beta-adrenergic agonist on the protein

Supplementation of β-AA in finishing beef cattle has been employed with the objective to increase feed efficiency, growth, body weight before harvest, and carcass weight (Beermann, 1993). Studies support these effects of β-AA on animal performance (Vasconcelos et al., 2008). Boyd et al. (1991) suggested that β-AA could increase lean muscle deposition by improving the utilization of amino acids for protein synthesis or by fluctuating the pattern of growth of the animal. Further, Brake et al. (2011) observed that the supplementation of β-AA decreased concentration of N in serum urea, Since that result suggests an effect on processes such as N retention and urea recycling, β-AA play a role in the variation of protein metabolism and proteolysis (Walker and Drouillard, 2010).

Data support a relationship between the effects of β-AA, such as RAC, and nitrogen source. The response of RAC was greater when animals were fed with ruminally degraded forms of nitrogen and RAC, illustrating that the type of protein (RDP and RUP) provided to the rumen microbiota may be important for optimizing the response of RAC in finishing cattle (Walker and Drouillard, 2010). It is important for future research to describe the influence of CP degradability in response to β-AA, as degradability is critical for protein metabolism and synthesis. By understanding these interactions, insight will be gained to improve diet formulation, taking full advantage of the action of β-AA and management in the finishing cattle industry (Samuelson K. L., 2016).

Effect of beta-adrenergic agonist on the bacteria population

Beta-adrenergic agonists as a synthetic catecholamine produce similar reactions compared to natural catecholamines, binding with beta-adrenergic receptors to increase lipolysis, gluconeogenesis, and glycogenolysis in adipose tissue and the liver (Beermann, 1993). Norepinephrine and epinephrine, which are natural catecholamines, have shown varied effects on bacteria, such as stimulating bacterial growth (Roberts et al., 2002), increasing the population of Gram-negative bacteria in *in vitro* experiments (Lyte and Ernst, 1992), as well as modifying gut motility and secretory response (Ruckebusch, 1983, McIntyre and Thompson, 1992). Some observed effects of adrenergic agonists include decreased frequency and intensity of rumen contractions, altered digestion of nutrients by ruminal microbes, impacts on eructation of gases from the rumen (Brikas et al., 1989, Leek, 2001). In an experiment with sheep, the amount of rumen glucose increased in response to β_2-adrenergic agonists when the animal consumed greater amounts of rapidly-fermented carbohydrates, predisposing the animal to acidosis (Aschenbach et al., 2002). Interestingly, researchers have also aimed to determine the effect of synthetic catecholamines, such as ractopamine hydrochloride (RAC), on the gut microbiome of livestock. Those results demonstrated that in sheep with oral inoculation of *E. coli* O157:H7, RAC increased pathogen proliferation in the cecum (Edrington et al., 2006).

In beef cattle, the supplementation of RAC with optimal concentrations of protein in the diet, according with the protein requirements of the animal, changed the microbial ammonia source, suggesting that the action of RAC can affect the breakdown of amino acids into ammonia by microorganisms in the rumen, and impact ruminal degradation of dietary (Walker and Drouillard, 2010). These results suggest an alternative option to increasing NPN in the diet as a source of

ammonia, using additional true protein to establish optimal ruminal fermentation in finishing cattle diets (Walker and Drouillard, 2010). In addition, natural catecholamines are involved in the greater affinity of bacteria to utilize iron, an essential mineral for bacterial growth (Kinney et al., 2000). Catecholamines increase Gram-negative bacteria in studies *in vitro* (Lyte and Ernst, 1992). Knowing that some of the bacteria present in the rumen for fermentation processes are Gram-negative (Walker and Drouillard, 2010), it is possible that the reaction of RAC as a synthetic catecholamine, could improve iron affinity to ruminal microbes, and increase bacterial population growth.

Conclusion

The beef industry in the US faces a challenge of increasing the supply of high-quality protein, while reducing negative impacts to the environment and maintaining the enterprise economy. Further, increases in feed efficiency of animals, are necessary to achieve improved beef production with limited resources. The gastrointestinal tracts of ruminants are capable of nutrients and producing energy from processes of the microbial community, which ultimately benefit the host. Changes in the diet are one of the most common factors that can impact the rumen microbial communities. New technologies, such as beta-adrenergic agonists (Ractopamine hydrochloride), which is used in the last weeks before harvest, have been developed to increase lean muscle deposition, improve gain, and enhance feed efficiency. As beta-adrenergic compounds have been shown to improve nitrogen retention, it is possible to affirm that they could influence protein metabolism and influence the rumen microbial communities. However, the effect of beta-adrenergic agonists (synthetic catecholamines) on the ruminal communities is relatively unknown. Natural catecholamines can impact bacteria growth, virulence factors, and adhesion; these results

are well-documented in studies *in vitro* and *in vivo* within different species. For this reason, potential effects of beta-adrenergic agonists on ruminal microbial communities exists. By understanding the response of the interaction between protein in different concentrations and beta-adrenergic agonists, researchers can further improve efficiency and animal growth, providing the tools to create diets that can maximize the response of those components, as well as identify how these interactions can impact the rumen ecosystem and the physiology of the animal.

CHAPTER TWO
EFFECTS OF PROTEIN CONCENTRATION AND BETA-ADRENERGIC AGONISTS ON RUMINAL MICROBIAL COMMUNITIES IN FINISHING BEEF HEIFERS

Abstract

To improve animal performance and modify growth by increasing lean tissue accretion, beef cattle production has relied on use of growth promoting technologies such as beta-adrenergic agonists. These synthetic catecholamines, combined with the variable inclusion of rumen degradable (RDP) and undegradable protein (RUP), may improve feed efficiency and rate of gain in finishing beef cattle. However, research about the impact of beta-adrenergic agonists and protein level and source on the ruminal microbiome is limited. The objective of this study was to determine the effect of different protein concentrations and beta-adrenergic agonist (ractopamine hydrochloride; RAC) on ruminal bacterial communities in finishing beef heifers. Heifers (*n*=140) were ranked according to body weight and assigned to pens in a randomized complete design to 6 different treatments, containing 3 protein treatments (Control: 13.9% CP, 8.8% RDP, and 5.0% RUP; High RDP: 20.9% CP, 13.4% RDP, 6.1% RUP; or High RUP: 20.9% CP, 9.1% RDP, 10.4% RUP) and 2 RAC treatments (0 and 400 mg/day). Rumen samples were collected from heifers by oral lavage 7 days before harvest. The DNA from the samples were sequenced to identify bacteria based on the V1-V3 hypervariable regions of the 16S rRNA gene using the Illumina MiSeq. Sequences and data from the treatments was analyzed using the R environment and PROC MIXED in SAS 9.4 (SAS Inst.; Cary, NC). Beta diversity was analyzed using PERMANOVA based on PCoA Bray-Curtis

distances and were significant among the protein and RAC treatments ($P < 0.05$). Alpha diversity metrics such as Chao1 and Shannon diversity indices were not significantly different ($P > 0.05$). Differences among treatments at variable taxonomic levels after analyses through DESeq2 were significantly different for the main effects of protein concentration ($P < 0.05$), rather than their interaction. These results suggest possible effects on the microbial communities with different concentration of protein, but limited positive impact with RAC. However, both may potentially act synergistically to improve performance in finishing beef cattle.

Introduction

The goal of the beef cattle industry is to increase lean mass yield, while also increasing feed efficiency. For this reason, in the past the ingredients and nutrient composition of finishing cattle diets have been altered by increasing the use of byproducts from the corn milling industry. These byproducts result in diets with excess supply of dietary protein (Samuelson K. L., 2016). Research has shown limited improvements in performance when crude protein (CP) concentration in finishing diets was greater than 13% (Galyean, 1996, Gleghorn et al., 2004). However, if CP was greater than 15.5% in combination of highly degradable protein in the rumen, results demonstrated a potential metabolic cost associated with ammonia detoxification. These results indicate that protein degradability plays an important role in animal performance (Lobley et al., 1995).

Producers and researchers further aim to develop new or improved technologies to manipulate the management and nutrition of finishing cattle with the intent to improve lean mass yield. Historically, one of these technologies involves the use of beta-adrenergic agonists such as ractopamine hydrochloride (RAC). Beta-adrenergic agonists (β-AA) are phenethanolamine compounds with similar characteristics to endogenous catecholamines, such as epinephrine and

norepinephrine (Bell et al., 1998). These β-AA have been demonstrated to improve feed efficiency, increase gain, as well as, decrease the deposition of fat in the carcass by the stimulation of adrenoreceptors localized in the muscle and adipose tissue (Vasconcelos et al., 2008, Montgomery et al., 2009). RAC, marketed under the commercial trade name Optaflexx (Elanco Animal Health. Greenfield IN), is a beta-adrenergic agonist (synthetic catecholamine) used to improve finishing cattle performance within the last 28 to 42 days prior to harvest (Avendaño-Reyes et al., 2006). The economic benefits of these beta-adrenergic agonists in the industry have been reflected in good reception of the supplement, reporting that 85% of feedlot operators use beta-adrenergic agonists during the finishing period (Samuelson, 2016).

The interaction between the RAC and dietary protein has been investigated, as some studies showed that animals fed diets high in ruminally degradable protein in combination with RAC, improved and increased the response compared with RAC alone (Beermann et al., 1986, Walker et al., 2006, Samuelson K. L., 2016). Given this information, researchers observed the interaction of RAC with ruminal microbes. Naturally occurring catecholamines have been shown to affect certain types of microorganisms, namely bacteria. Walker et al. (2006) suggested that the supplementation with RAC could alter proteolysis processes in the rumen, affecting the potential of the rumen microbiota to utilize ruminal degradable protein, and observed that the ratio between protein fractions (Ruminal degradable protein (RDP) and Ruminal undegradable protein (RUP)) could increase the response to RAC. Therefore, it was hypothesized that the interaction of various concentrations of degradable vs. undegradable protein in combination with beta-adrenergic agonists may affect the ruminal microbial communities in beef heifers during the finishing period.

The objective of this study was to determine the effect of protein concentration and beta-adrenergic agonist (RAC) supplementation on ruminal microbial communities in finishing beef heifers.

Methods and Materials

Animal Management

This study and all procedures were approved by the New Mexico State University Institutional Animal Care and Use committee. Calves were weighed using a Daniels Bud Box System (AH- 10; Daniels Mfg., Ainsworth, NE). Animals were vaccinated and oral parasiticide was provided (Safeguard; Intervet Inc., Millsboro, DE). Calves were fed a commercial starter diet once daily. Prior to the study initiation, a total of 140 heifers were weighed individually (423 ± 1.8 kg) and ranked by body weight. Based on body weight, animals were separated in 4 blocks and assigned to pens with similar average weight and standard deviation. Heifers were vaccinated again (Vista 3; Merck Animal Health, Summit, NJ) and a commercial growth implant was administered (Revalor-200; Merck Animal Health).

Experimental Design

The study utilized a randomized complete block design (RCBD) with sampling and repetitions, consisting of 48 pens and 4 blocks (12 pens per block with 3 heifers per pen). Within each block, pens of heifers were randomly assigned to 1 of 6 treatments in a 2 x 3 factorial arrangement. The treatments consisted of 400 mg or 0 mg of ractopamine hydrochloride (RAC; Optaflexx, Elanco Animal Health, Greenfield IN) per head per day supplied in steam-flaked corn-based diets with 3 different dietary protein treatments (**Table 2.1**): **CON** (13.9% PC, 8.8% RDP, and 5.0% RUP),

Table 2.1 Ingredients and nutrient composition of diets fed to finishing heifers.

Ingredients	Treatments[2]		
	CON	High RDP	High RUP
Ingredient. % of DM			
Corn grain, flaked	67.1	57.0	57.0
Wet corn gluten feed	18.0	18.0	14.5
Corn Stover	9.00	9.00	9.00
Soybean Meal	---	9.60	---
Corn gluten meal	---	---	14.50
Corn oil	0.90	1.40	---
Urea	1.01	1.49	---
Supplement[3]	3.99	3.51	5.00
Nutrient Analysis, DM basis			
TDN, %	84.4	84.3	84.2
CP, %	13.9	20.9	20.9
DIP, %	8.85	14.40	9.70
UIP, %	5.05	6.46	11.18
ADF, %	8.80	8.97	9.10
Ca	0.78	0.76	0.79
P	0.41	0.45	0.44
Mg	0.19	0.22	0.18
K	0.75	0.85	0.78
S	0.17	0.20	0.30
Na	0.13	0.14	0.15
Zn	79	80	80
Fe	254	251	228
Mn	49	51	48
Cu	13.70	13.50	15.70
ME[4]	3.05	3.05	3.04
NEm, Mcal/kg[5]	2.08	2.07	2.07
NEg, Mcal/kg[6]	1.41	1.41	1.40

[1] Information from (Samuelson K. L., 2016)
[2] Treatments were in a 2 x 3 factorial arrangement with sampling and repetitions, with 2 levels of ractopamine hydrochloride and 3 dietary protein treatments.
[3] Contain dried distillers' grains with soluble, limestone, salt, trace minerals (1.8% Cu, 9.0% Zn, and 360 ppm Se; Beefmax 0510; Cargill Inc.) vitamins (1030 IU vitamin A, 500 IU vitamin D, and 5.62 IU vitamin E per kg of DM) and medicated supplement (supplied 33mg of monensin and 9.8mg of tylosin per kg of dietary DM; Elanco Animal Health, Indianapolis, IN).
[4] $ME = TDN \times 4.409 \times 0.82$ (NRC, 1996).
[5] $NEm = 1.37 \times ME - 0.138 \times ME^2 + 0.0105 \times ME^3 - 1.12$ (NRC, 1996).
[6] $NEg = 1.42 \times ME - 0.174 \times ME^2 + 0.0122 \times ME^3 - 1.65$ (NRC, 1996).

High RDP (20.9% CP, 13.4% RDP, 6.1% RUP) and **High RUP** (20.9% CP, 9.1% RDP, 10.4% RUP). After sorted into pens and weighed (initial BW= 498 Kg (Block 1), 527 Kg (Block 2), 551 Kg (Block 3) and 575 ± 3.82 Kg (Block 4)), heifers were start to be fed the dietary treatments. With a treatment period of 35 days, the animals were fed twice daily. Prior to the treatment period, heifers were fed a standard feedlot finishing diet.

The RAC (Optaflexx; Elanco Animal Health, Greenfield IN) treatment was administrated on top of the protein treatments in feed bunks once daily according with the doses of RAC (0 or 400 mg) per heifer assigned to 1 of 3 dietary protein treatments. The RAC was mixed with 150 g of wet corn gluten feed (Sweden Bran; Cargill Inc.) for pens receiving RAC treatment, whereas pens without RAC treatment were top dressed with 150 g of wet corn gluten feed only.

Rumen sampling from the finishing heifers

Rumen fluid samples were collected from the 140 heifers by oral lavage at day 28 (7 days before harvest) with a metal suction strainer. Rumen sampling was carried out 4 hours after morning feeding and deposited in a stoppered side-arm flash. To prevent saliva contamination, the first 100 mL of the rumen fluid was discarded. Further, each sample was mixed in a beaker, rumen content pH was determined using a handheld pH meter, and the samples were divided into 15 mL aliquots. The samples were stored at -20ºC until further analysis.

DNA Extraction for Ruminal Microbial Communities

Rumen samples were processed via DNA extraction and purification at the University of Tennessee, Knoxville, TN. DNA was extracted from the rumen fluid using the repeated bead beating plus column method described by Yu and Morrison (2004). The rumen samples from the

15 mL aliquots were used for distribution of new 0.2 g samples. The 0.2 g sample was added to a 2 mL beaded screw cap tube, containing 0.5mm ZR BashingBead lysis matrix for cell lysis (Zymo Research, Irvine, CA, USA). A total of 1 mL of lysis buffer (500mM NaCl, 50mM tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate) was added, contents were homogenized for 3 minutes at 21 Hz, and tubes were incubated at 70°C for approximately 15 minutes at 16,000 x g. After centrifugation and incubation, the supernatant was transferred to 2 mL tubes and 300 µL of lysis buffer was added to the original sample again for the repetition of the previous step. Following the repetition, the supernatants from individual samples were pooled.

Nucleic acid precipitation was conducted by the addition of 260 µL of 10 M ammonium acetate in each tube. Samples were then mixed and incubated for 5 minutes on ice. Following the incubation, the tubes were centrifugated at 4°C for approximately 10 minutes at 16,000 x g. The supernatant was distributed into two tubes of 1.5 mL each, for the addition and mixture with one volume of isopropanol. After 30 minutes of incubation on ice, the isopropanol precipitation was observed. The samples were centrifuged at 4°C for 15 minutes at 16,000 x g, the supernatant was discarded, and the presence of a nucleic acid pellet in the bottom of each tube was confirmed. Nucleic acid pellets were washed in 70% ethanol and dried for 3 minutes. The pellet was then dissolved in 100 µL of Tris-EDTA buffer and pooled for individual samples.

The QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) was used for purification. Contamination of RNA were removed by the addition of 2 µL of DNase-free RNase (10 mg/mL) and incubated at 37°C for 15 minutes. To remove protein contamination, 15 µL of proteinase K and 200 µL of buffer AL were added. Samples were then incubated for 10 minutes at 70°C. After

incubation, the samples were mixed with 200 µL of 100% ethanol. The final samples were transferred to QIAamp columns (QIAGEN, Valencia, CA, USA) and centrifugated at 16,000 x g for 1 minute. Flow through was rejected and a washing process was repeated by the addition of Buffer AW1 and AW2 and centrifuged after each addition under the same conditions. The column was centrifugated at room temperature at 16,000 x g for 1 minute to obtain a dry column. Following the drying process, 70 µL of Buffer AE were added to the column membrane and the samples were incubated for 2 minutes at room temperature. A volume of 30 µL of Buffer AE was added again to the column membrane and incubated again under the same previous conditions. To elute DNA, QIAamp column was placed into a 1.5-mL tube and centrifuged for 1 minute. Finally, the DNA samples were kept at -20°C for subsequent amplification and library preparation processes.

DNA Amplification for Ruminal Microbial Communities

Amplicon libraries of the 16S rRNA gene for bacteria (V1-V3) were constructed as previously described (Kozich et al., 2013), using primers 27F (Stahl, 1991) and 519R (Lane et al., 1985). Each 20 µL polymerase chain reaction [PCR] amplification reaction contained 0.5 µL Terra PCR Direct Polymerase Mix (0.625 Units), 7.5 µL nuclease-free sterile water, 10 µL 2X Terra PCR Direct Buffer, 1 µL indexed fusion primers (10 µM), and 1 µL DNA (20 to 70 ng). The thermocycling conditions were: 3 minutes of initial denaturation at 98°C, 25 cycles including 30 seconds at 98°C, 30 seconds at 55°C, and 45 seconds at 68°C. The final extension was conducted for 4 minutes at 68°C. The primers utilized for DNA amplification are listed in **Table 2.2**.

Once DNA amplification was completed, the products of PCR from each sample were normalized (1 to 2 ng/ µL) using the Just-a-Plate ™ 96 CPR Purification and Normalization kit (Charm Biotech, MO, USA) as described by the manufacturer.

Table 2.2 DNA PCR amplification primers

	Regions	**Primers**	**Sequences**	**Source**
Bacteria	V1 – V3	27F	KRGTTYGATYNTGGCTCAG	(Stahl, 1991)
		519R	GWRTTACCGCGGCKGCTG	(Lane et al., 1985)

After the normalization, the libraries were pooled in a 10 μL per sample and purified using the Nucleospin® Gel and PCR Cleanup kit (Takara Bio USA, Inc., Mountain View, CA) following the manufacture's protocol.

Libraries were analyzed for quality utilizing the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and DeNovix QFX Fluorometer (DeNovix dsDNA Fluorescence Quantification Assay) for quantification. Libraries were sequenced using the 2x250, v2 500-cycle kit and Illumina MiSeq sequencer (lllumina, San Diego, CA, USA).

DNA Amplicon Sequence Data Processing for Ruminal Microbial Communities

Bacteria communities sequenced by the Illumina MiSeq 2x250 and resultant fastq files were processed through an R pipeline as described by Callahan et al. (2016b). The files were introduced into R software and open-sourced R packages 'phyloseq' (McMurdie and Holmes, 2013) and 'DADA2' (Callahan et al., 2016a) for filtering, merging, and taxonomy assignment. Forward reads were trimmed based on quality score ($Q \geq 25$) and the error expected per each read was set to two for forward reads, filtering any data which did not meet these criteria (Edgar and Flyvbjerg, 2015). Divisive Amplicon Denoising Algorithm 2 [DADA2] was used for accuracy in the identification of real variants, and amplicon error correction used a naïve Bayesian classifier (Wang et al., 2007, Callahan et al., 2016a) Amplicon sequence variants [ASVs] were generated from DADA2, with the purpose of discreetly increasing genetic resolution in comparison to 97% operational taxonomic units [OTUs] (Callahan et al., 2017, Glassman and Martiny, 2018). Filtered forward reads were merged and chimeras were removed. Silva v132 database (Quast et al., 2012) was used for taxonomic assignment at the genus level. Protozoa and Archaea were removed from the data.

Statistical Analyses

Alpha-diversity and beta-diversity metrics were analyzed using R. Alpha-diversity was calculated utilizing observed richness [Observed]; expected richness [Chao1]; and richness and evenness estimates were completed by Shannon. Alpha diversity was calculated for bacteria and the differences among treatments were analyzed by SAS v9.4 (SAS Institute, Cary, NC, USA). Variables were analyzed using a MIXED procedure, with significance determined at $\alpha = 0.05$. Beta-diversity measures utilized a Bray-Curtis distance matrix to produce principal coordinates analyses [PCoA]. Subsequently, PERMANOVA was utilized with 999 permutations to establish significance of Bray-Curtis PCoA by the use of 'vegan' in R (Oksanen, 2011). The abundance differences among the treatments for bacteria communities were calculated individually utilizing R package 'DESeq2' (Anders and Huber, 2010, Love et al., 2014). DESeq2 package uses data from taxa tables and raw count information to develop an internal normalization of the communities. The internal normalization is performed by the calculation of a geometric mean across all samples, then, the counts for each gene in each sample is divided by the mean. Significantly, DESeq2 package works to correct RNA composition bias, library size, targeting small genes that could be expressed in some samples but not in others, and is used to calculate differential abundances of the microbial communities. DESeq2 uses a depreciation estimation for dispersions and ford changes to account for replicates. The outliers produced during the process were removed automatically by the use of Cook's distance, as well as the genes that could not meet the threshold of normalized counts were removed with the purpose to improve the detection of the power present in DESeq2.

Results

Finishing heifer performance

Samuelson K. L. (2016) reported that the performance on body weight and carcass-adjusted final body weight of the heifers supplemented with RAC were significantly greater ($P < 0.01$) than animals on the control. Average daily gain (ADG) in heifers receiving RAC was greater ($P < 0.01$) compared with heifers not receiving RAC. The ADG with RAC with different protein concentration was 1.22±0.02 kg (RAC - Control), 1.26±0.02 kg (RAC – High RDP) and 1.36±0.02 kg (RAC – High RUP) ($P < 0.01$), compared with animals not receiving RAC (0.931±0.02 kg Control, 0.949±0.02 kg High RDP and 0.979±0.02 kg High RUP) ($P > 0.05$).

Sequence Data and diversity of rumen bacterial communities

After complete quality control, filtering, and processing the sequences in R software, the sampled ruminal contents of 140 finishing beef heifers were identify 13,805 unique sequences. Alpha and beta- diversity measures indicated the richness, evenness, and diversity among the bacterial species. The sequence reads were examined via observed richness and Chao1 to calculate the observed and estimated richness, respectively. Shannon diversity indices were analyzed to determine the bacterial richness and evenness. The data representing bacterial community richness did not indicate any difference ($P > 0.05$) among the treatments. The alpha-diversity indices for each treatment was similarly not significantly different. (**Figure 2.1**).

Data was reduced and analyzed by principal coordinates analyses (PCoA) to determine separation into sample clusters. PCoA was achieved using the Bray-Curtis distance method. Bray-Curtis Distance method is a beta-diversity measure that identifies differences among microbial

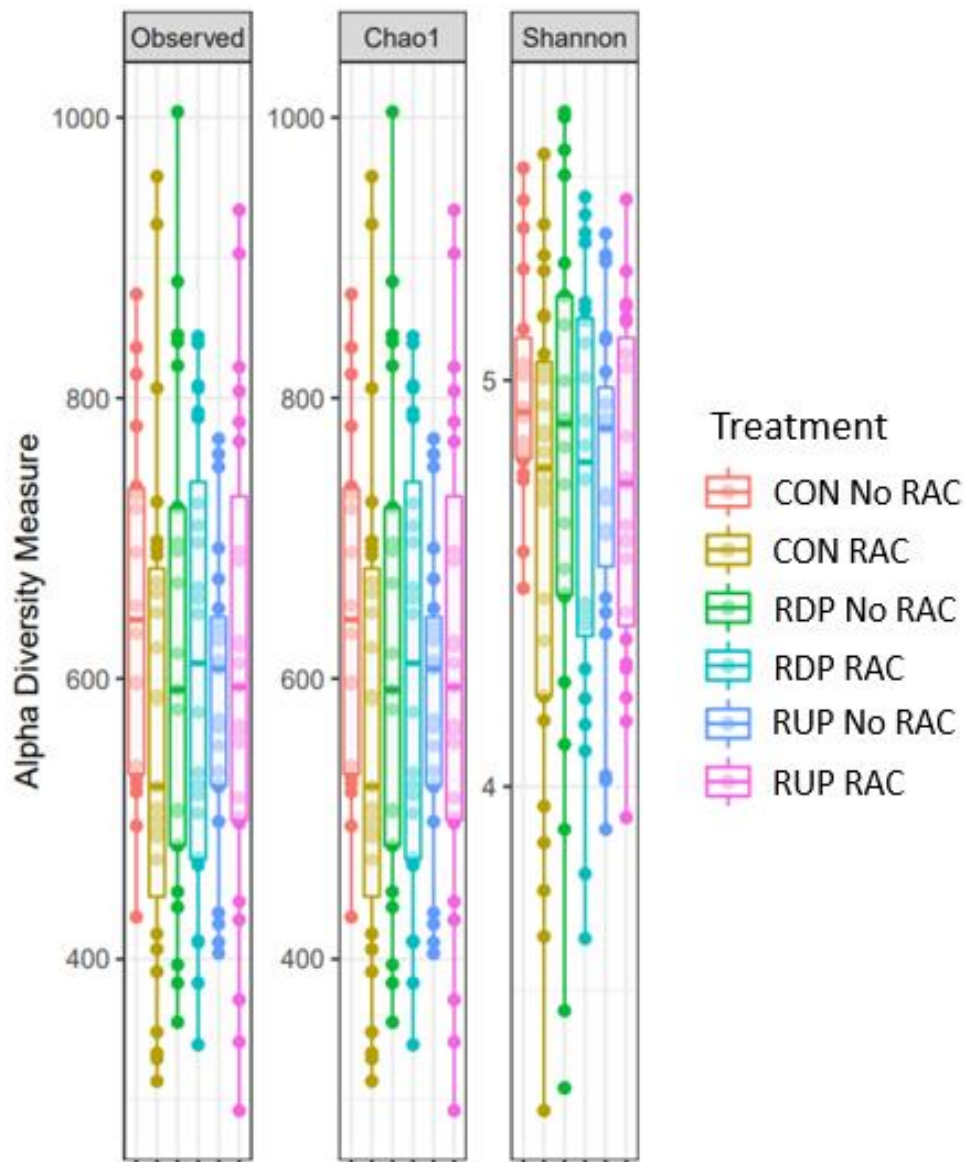

Figure 2.1 Alpha-diversity metrics for ruminal bacterial richness and evenness among the treatments described by Observed, Chao1 and Shannon. (CON) Control, (RDP) Ruminal degradable protein, (RUP) Ruminal undegradable protein, (RAC) Ractopamine hydrochloride.

community taxonomic composition (Lozupone and Knight, 2005) (**Figure 2.2**). Visually, there was no indication of sample clustering and indicated some degree of treatment overlap. PERMANOVA was utilized to analyze beta-diversity, showing differences among the 6 treatments ($P < 0.001$). However, this was likely due to differences in the dispersions among samples. Statistical analyses and PCoA visualization do not always correspond if there is a weak separation of treatment groups. The PCoA visualization is only capturing approximately 18.6% of the variation. Thus, differences may be more evident on a different axis.

Bacterial Community Composition

The abundances of the top 10 phyla present in rumen bacterial samples revealed the most predominant bacterial phyla identified in the samples within each treatment and interaction was Bacteroidetes (30-35%) and Firmicutes (57-60%), showing similar results as Jami et al. (2014) studies of rumen microflora (**Figure 2.3**). The remaining phyla in the rumen bacterial samples represented less than 1% of the remaining cleaned reads. Patescibacteria and Actinobacteria accounted for the less dominant phyla (greater than 0.5% but less than 1%). At the genus level, the most abundant genera were primarily of Prevotella_7 and Prevotella (16.4% and 8.1% respectively) followed by *Lachnospiraceae NK3A20 group* and *Erysipelotrichaceae UCG-002* (8.5% and 7.6% respectively) (**Figure 2.4**). The relative taxonomy abundance analysis was analyzed to reflect the mean of the relative abundance (reads of the taxon/total reads in the sample) among the 6 treatments. The differential abundance based on dietary treatment (2×3 factorial design) conducted by DESeq2 in R did not show significant differences at the phylum level.

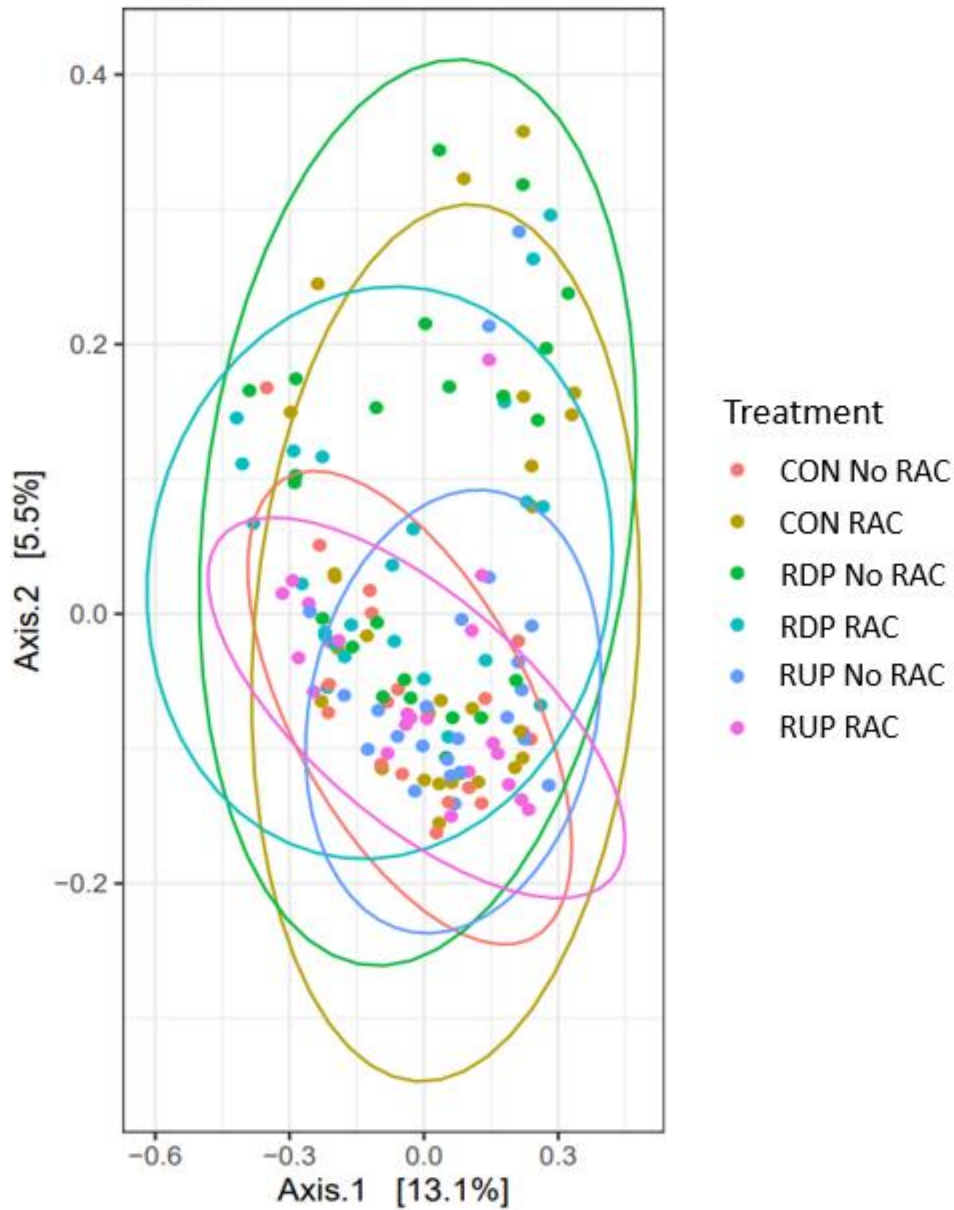

Figure 2.2 Beta-diversity measurement for bacterial communities using Bray-Curtis PCoA and 999 permutations grouped by treatments. Ellipse and points with same color represent taxa within treatments groups ($P < 0.05$). Clusters indicate similarities of taxa among treatments groups. (CON) Control, (RDP) Ruminal degradable protein, (RUP) Ruminal undegradable protein, (RAC) Ractopamine hydrochloride.

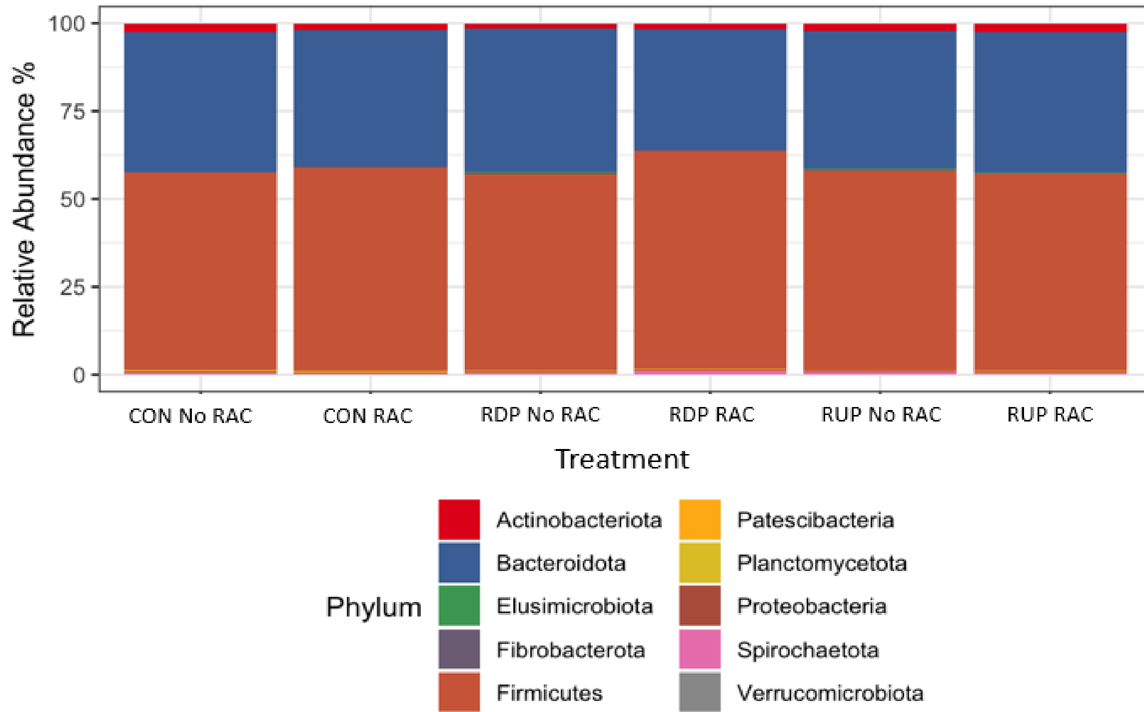

Figure 2.3 Overall phyla relative abundance identified in the ruminal bacterial communities. (CON) Control, (RDP) Ruminal degradable protein, (RUP) Ruminal undegradable protein, (RAC) Ractopamine hydrochloride

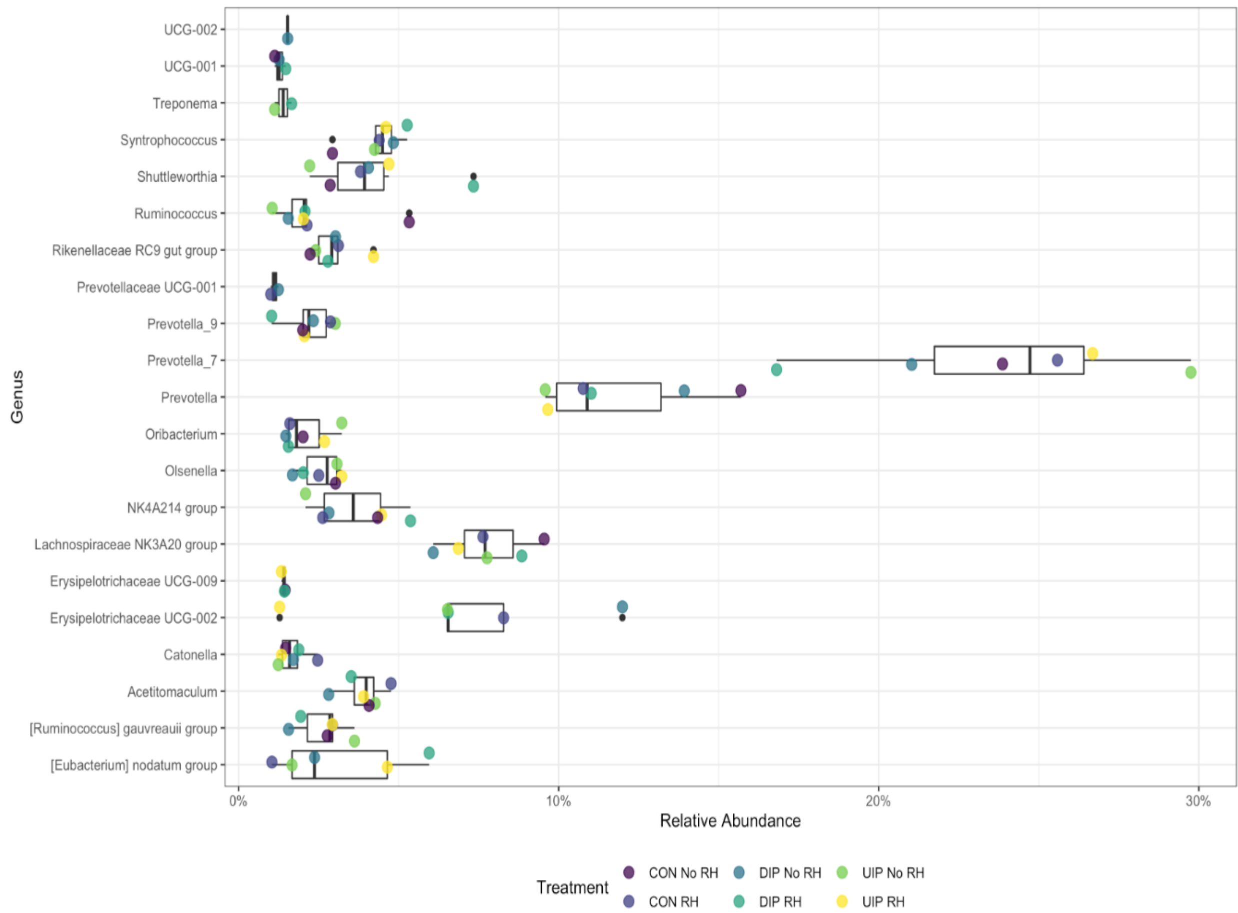

Figure 2.4 Taxonomic profile of the 99% genera relative abundance of the ruminal bacterial communities among the treatments.

Genera abundances were not different across the treatments for the interaction of protein and RAC (*P* > 0.05); however, there were differences when examining the main effects of protein and RAC (**Figures 2.5 and 2.6**). Genera *Ruminococcus gauvreauii group* (*P*=0.005), *UCG_001* (*P*=0.04), *UCG_002* (*P*=0.015), *Ruminococcus* (*P*=0.04) and *Eubacterium nodatum group* (*P*=0.02) indicated significant differences in abundance between protein concentrations (**Table 2.3**). *Eubacterium nodatum group* showed significant differences in abundance with the RAC treatments (*P*=0.016). (**Table 2.4**).

Discussion

The extensive use of beta-adrenergic agonists in the beef industry has resulted in positive improvements in the efficiency of body weight gain and, carcass characteristics, and improving the competitiveness of the US beef industry (Avendaño-Reyes et al., 2006, Stackhouse-Lawson et al., 2013). Samuelson K. L. (2016) reported that heifers of this study supplemented with RAC had greater final body weight (1.9% increase) and ADG (42.9% increase) contrasted to animals without supplementation. The ADG results observed by Samuelson K. L. (2016) were greater than results reported by Abney et al. (2007), where animals received just 200mg of RAC daily. Due to the increase of ADG in heifers with the RAC treatment, those animals also increased the requirements of metabolizable protein as the consequence of increasing the gain requirements.

The composition of the diet and the different levels of intake are the main effects that cause variations in the metabolizable energy, generating a direct effect in the gain composition (Fox and Black, 1984, Church, 1993). However, animals with and without RAC treatment had a positive balance of metabolizable protein supplied by the diet, confirming that the metabolizable protein was the correct amount to meet the dietary CP requirements for the performance of those animals.

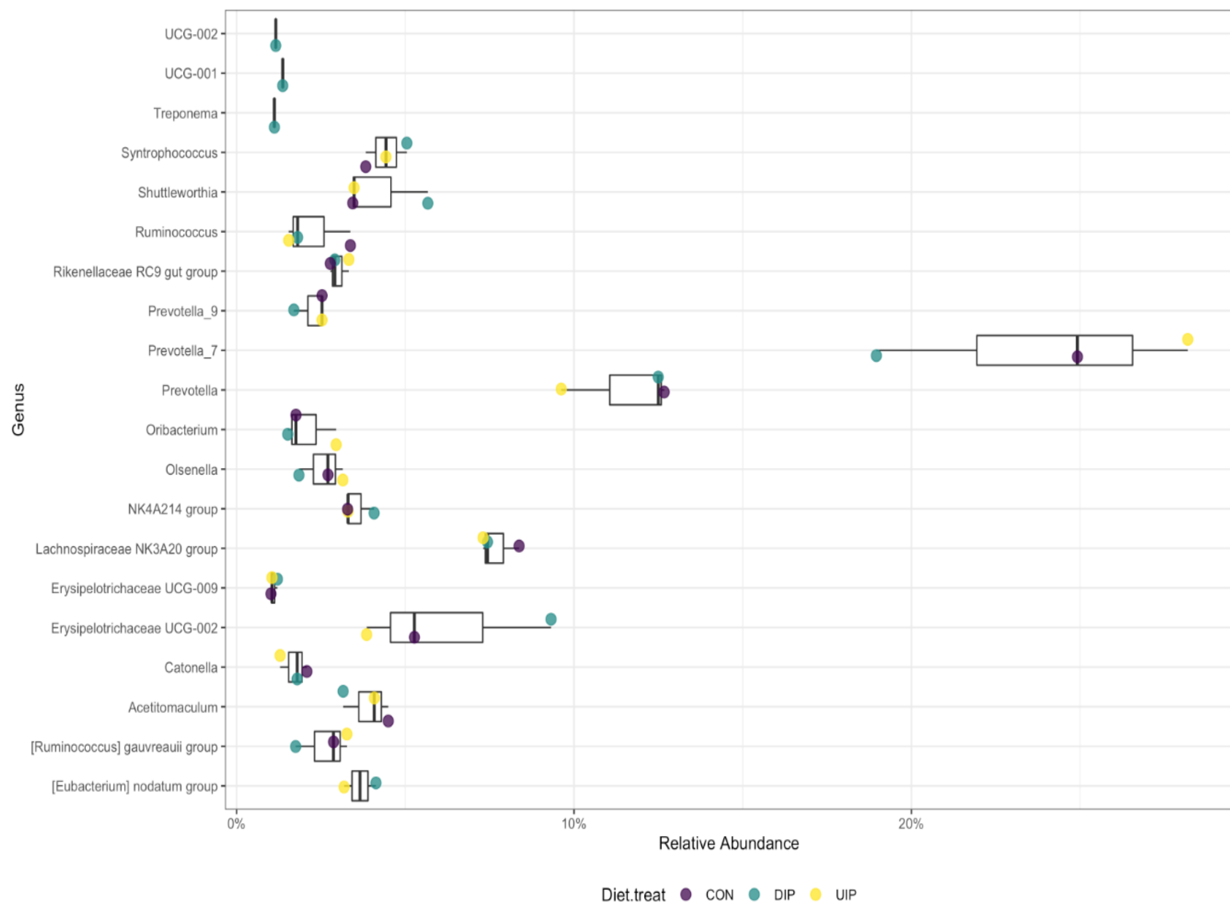

Figure 2.5 Taxonomic profile of the 99% genera relative abundance of the ruminal bacterial communities between the protein concentration

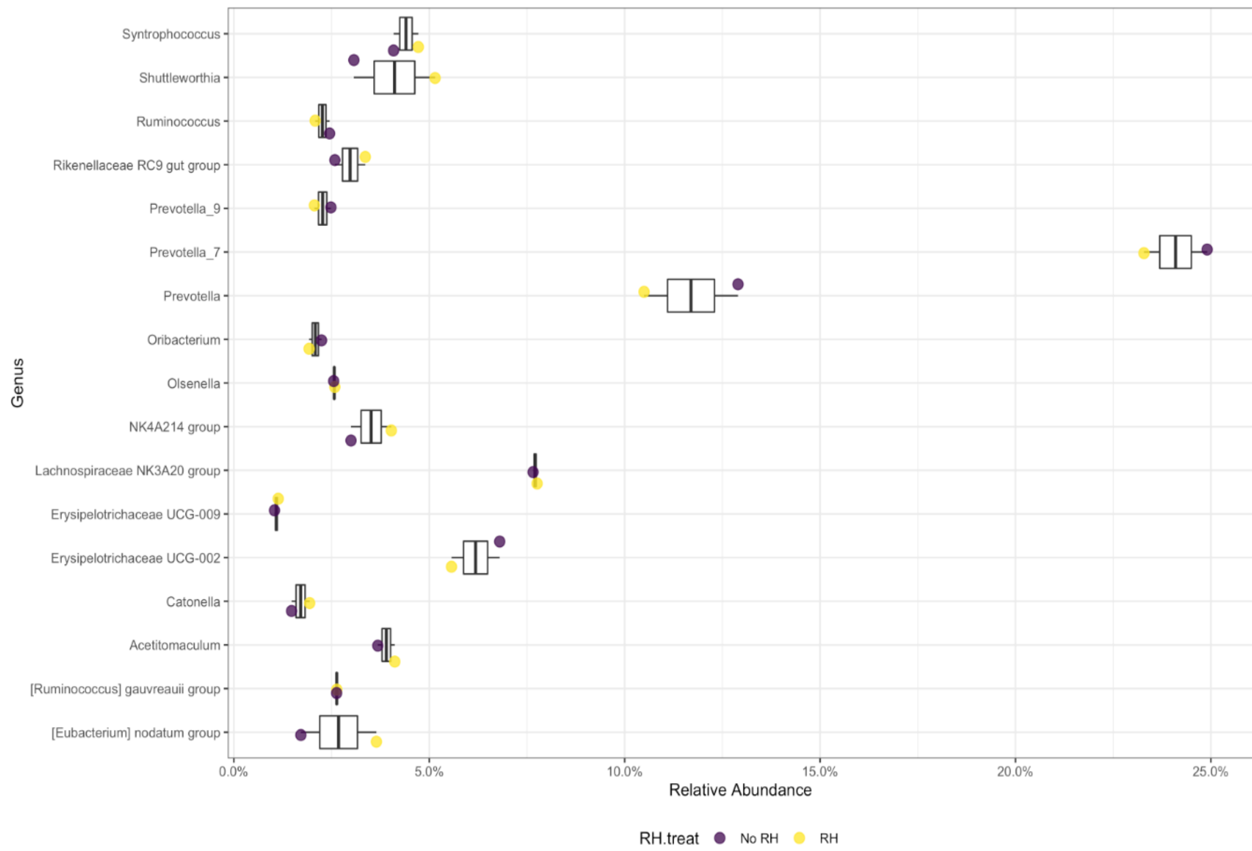

Figure 2.6 Taxonomic profile of the 99% genera relative abundance of the ruminal bacterial communities between the RAC concentration

Table 2.3 Percentage relative abundance of significant genera among protein concentration (CON, High RDP and High RUP).

Genus	Protein Treatments[1]			SEM	*P* value[2]
	CON	RDP	RUP		
Eubacterium nodatum group	0.7341	2.995	2.225	0.005	0.021
Ruminococcus gauvreauii group	1.972	1.26	2.216	0.0017	0.005
Ruminococcus	2.591	1.374	1.049	0.0046	0.041
UCG - 001	0.5735	1.063	0.1729	0.0026	0.045
UCG - 002	0.3961	0.4194	0.8574	0.0012	0.015

[1]Values indicate the percent (%) relative abundance
[2]Significance difference determined at $P<0.05$.

Table 2.4 Percentage relative abundance of significant genera between RAC concentration (0 mg and 400 mg).

Relative genus abundance	RAC treatment[1]		SEM	*P* value[2]
	RAC	No RAC		
Eubacterium nodatum group	2.781	1.189	0.004	0.016

[1]Values indicate the percent (%) relative abundance
[2]Significance difference determined at $P<0.05$.

Ruminal degradable protein (RDP) and ruminal undegradable protein (RUP) are different fractions of dietary protein supplied in the diet of ruminants. The site of digestion of those protein sources are different: RDP will be degraded by ruminal microorganisms, whereas RUP will escape the rumen to be digested and absorbed in the abomasum and small intestine (Bach et al., 2005). In this study, some genera were significantly different between the protein concentrations (CON, High RDP and High RUP) in the diet (*Ruminococcus, Ruminococcus gauvreauii group, Eubacterium nodatum group, UCG-001 and UCG-002*). These different cellulolytic groups are a well-known bacterial groups present in the rumen of ruminants (Russell et al., 2009) that participate in fiber digestion (Forsberg et al., 1997). Those results were not surprising since observations have shown that increasing dietary protein increases highly fermentable substrates, decreases the diversity of the rumen microbes, and increases the efficiency of those microbes to utilize these substrates and dominate the rumen microbial structure (Fernando et al., 2010).

Studies from Walker et al. (2006) and Beermann et al. (1986) demonstrated that beta-adrenergic agonists produced a reaction in ruminants fed with dietary crude protein fractions(RDP and RUP), due to the more easily-degraded protein metabolized by ruminal microbial communities, increasing nitrogen retention and uptake of amino acids. Therefore, it was hypothesized that beta-adrenergic agonists and different concentrations of dietary crude protein fractions (RDP and RUP) may affect the ruminal microbial communities in finishing beef heifers. Ruminal microbes have been reported to impact feed efficiency in ruminants by the conversion of nutrients to energy that can be used by the animal host (Myer et al., 2015). The fermentative products produced by the rumen microbes supply energy to the animal, and this energy is used in metabolic processes within the animal. In this study, the bacterial communities of the rumen did not differ among the

interaction of RAC and protein concentration. Specifically, there were limited impacts from RAC supplementation. No significant differences were found when examining alpha-diversity (within-sample diversity). However, beta-diversity (between samples diversity) results showed significant differences among the dietary crude protein (CON, High RDP and High RUP) and RAC. However, this difference was likely observed due to the wide distribution among the treatments. The microbial communities of this study were similar in microbial composition to existing research, such as those from Duffy (2019) and Paz et al. (2016) where *Bacteroidetes* and *Firmicutes* were the most abundant phylum among the treatments. The study from Myer et al. (2015) affirm that the greater abundance of *Bacteroidetes* and *Firmicutes* over other phylum were connected with the increase of ADG of those animals and could affect feed efficiency. These results suggest that the addition of beta-adrenergic agonists in the diet may interact with phyla with the objective to improve the performance of the animal.

The RAC has an affinity for beta-1 and beta-2 adrenergic receptors. Receptors B_1-AR are found prominently in the heart, while B_2-AR are present in the vascular smooth muscle, the skeletal muscle, in the walls of the gastrointestinal tract, the bladder, and in the bronchioles. The action of beta-adrenergic receptors binding with a beta-adrenergic agonist may intensify the process of muscle hypertrophy, due to the increase of blood flow to the skeletal muscle. These reaction on of blood flow improve the delivery of energy sources (ATP from the mitochondria) and substrates (amino acids) required for the synthesis of protein. In addition, in the adipose tissue may aid the transportation of non-esterified fatty acids away from adipose tissue increasing the degradation of lipids (Mersmann, 1998). The increase of blood flow also can augment heart rate and the circulation of different endocrine hormones, such an insulin which is an anabolic hormone,

generating significant effects on muscle protein metabolism. However, the effect on blood flow the beta-adrenergic agonists has is short duration. So, it is unlikely that the processes of muscle hypertrophy are the sole result of the changes in blood flow.

Different studies confirm an effect of adrenergic agonists on the contractions in the rumen (Ruckebusch, 1983, Brikas et al., 1989, Leek, 2001). Adrenergic agonists reduce the frequency and the intensity of the ruminal contractions, producing an effect on the digestion of the nutrients from the diet by the ruminal microbial community (Brikas et al., 1989, Leek, 2001). Normally, this effect is caused by alpha-adrenergic agonists, such a phentolamine, which present an affinity with alpha-adrenergic receptors (α-AR). By the activation of these α-AR by the α-AA, the receptors produced and inhibitory effect, releasing acetylcholine from the parasympathetic postganglionic nervous terminals. The sympathetic system response of these reaction is an indirectly inhibition of the gastrointestinal functions and motility (Costanzo, 2013). Additionally, reduce the frequency and amplitude of reticulum and rumen dorsal sac contraction, decreasing the diameter of the arteriole and increasing the resistance to blood flow. These receptors are present on the arterioles of the skin and the splanchnic vasculature (Costanzo, 2013). In this study, we supplied in the diet a beta-adrenergic agonist, RAC, which as mentioned above, has an affinity for beta adrenergic receptors, suggesting that the reason we may not have observed any effect of RAC in the rumen could be explained by the affinity of the receptor.

Beta-adrenergic agonists share similarities with natural catecholamines in structural and pharmacological properties (Beermann, 1993). Catecholamines, such as norepinephrine, epinephrine, and dopamine, produced an increase in Gram-negative bacterial growth (Lyte and Ernst, 1992). The demonstration of the catecholamines ability to interact and impact bacterial

growth has been studied by the examination of different mechanisms. Lyte and Bailey (1997) showed that the position of the hydroxyl group on the catechol ring induced bacterial growth under specific conditions of iron restriction. Iron requirements play an important role in microbial and mammalian physiology. For this reason, bacteria under poor iron environment due to the presence of lactoferrin (iron-sequestering glycoproteins) and transferrin, localized in the mammalian gut lumen, and in the plasma and throughout all internal organs respectively, will secrete siderophores and bind with the hydroxyl group of the catecholamines to obtain the iron required for growth. Freestone et al. (2000) confirmed that catecholamines, such as norepinephrine, facilitate the extraction of iron from transferrin and lactoferrin for the availability of the bacteria utilization for growth. Due to the demonstrated effects of natural catecholamines on bacterial growth, this study hypothesized that beta-adrenergic agonists, as a synthetic catecholamine, could impact the rumen microbial communities. However, in this study there was no impact of RAC on the rumen microbial communities. Although the supplementation with RAC or different protein sources, such as RDP and RUP, altered specific microbes of the rumen bacteria composition, the overall microbial population was not affected by the experimental treatments. The lack effect of beta-adrenergic agonists and the rumen microbes may be due to absorption. The oral administration of RAC results in the absorption from the gastrointestinal tract by the B2-AR, with a significant portion reaching systemic circulation. In cattle and monkeys, this amount reaching circulation is approximately >45%, whereas in swine, it has been documented to be >85% (Ungemach, 2004). These circulation data suggest that beta-adrenergic agonists may have the same characteristics as the RUP, which escape the rumen and are digested and absorbed in the small intestine. Then the administration of β-AA will not interact of affect the microorganisms in the rumen, and its scape

for absorption in the small intestine. Subsequently in the hindgut, RAC may play the same role as catecholamines with regard to bacteria and the necessity of those to obtain iron for its growth. Further, beta-adrenergic agonists are lipophilic, presenting aliphatic amine compounds with an alkaline pKa ranging from 9.4 for RAC. However, at physiologic pH, RAC is ionized by protonation and more than the 99% will be positively charged at a pH closer to 7.4, creating a non-lipophilic compound that would be activated just with the specific receptor (Ungemach, 2004). The absorption location of the beta-adrenergic agonists is not clear. Different types of beta-adrenergic agonists have shown absorption in the small intestine and less absorption in the stomach in experiments with rats and absorption in the duodenum in humans. However, due to the limited information published about the absorption side of the beta-adrenergic agonists in ruminants *in vivo*, it is possible to hypothesize that the site of the primary absorption of beta-adrenergic agonists in ruminants is in the small intestine. This absorption could be through = passive diffusion, as a result of the neutral pH in the small intestine preventing beta-adrenergic agonists to form cations at the phenethanolamine nitrogen, increasing the absorption through the intestinal mucosa (Smith, 1998).

The results in this study suggests that the action of RAC and different concentration of protein to improve gain and muscle growth may not be synergistic, opening the idea that the supplements functions independently. Therefore, knowing the action of beta-adrenergic receptors in the regulation of gastrointestinal functions, such as the secretion of bicarbonate and acid, intestinal motility, and gastrointestinal mucosal blood flow, future studies should observe the relationship of beta-adrenergic agonists with the beta-adrenergic receptors present in beef cattle's gastrointestinal tract and in the rumen to obtain a better understanding of the mediation of beta-adrenergic

receptors in bovine physiological responses (Canfield and Paraskeva, 1992, Anthony, 1996, Thollander et al., 1996).

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VITA

Alison Paola Pfau was born June 2nd, 1990 in Bogota Colombia. Alison attended the Universidad Nacional de Colombia to receive her bachelor of Animal Science in March 2017. By the coordination of The Ohio International Agricultural & Horticultural Intern program, Alison was an intern in one of the most important organic dairy farms in US (Aurora Organic Dairy). There she worked as an assistant in different areas, such a raising calf, assisting deliveries of cows in the last third of pregnancy, and as a breeding assistant in the production area.

Since then, Alison has been working in many facets of the dairy industry. She worked at the Dairy Authority Lab, LLC, testing different dairy farm's milk with the aim of offering diagnostics for large animal diseases and milk quality. Further, she worked in different conventional dairy farms as a head herdsman assistant and manager, coordinating and helping in the daily handling of cattle, including transport, administration of medicine, breeding assistance in artificial insemination of dairy cows, and management of the entire dairies.

Alison began her Master of Science in Animal Science at the University of Tennessee in Fall 2020. Alison worked with Dr. Phillip Myer for her Master of Science in the Animal Science department, and graduated with her degree in Spring 2022.