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Mechanisms of Programmed Nutrition in Finishing Cattle *in Vivo* and Ruminal Parameters of Amaize Supplementation *in Vitro*

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MECHANISMS OF PROGRAMMED NUTRITION IN FINISHING CATTLE *IN VIVO* AND RUMINAL PARAMETERS OF AMAIZE SUPPLEMENTATION *IN VITRO*

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment at the
University of Kentucky

By

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Lexington, Kentucky

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2022

ABSTRACT OF DISSERTATION

MECHANISMS OF PROGRAMMED NUTRITION IN FINISHING CATTLE *IN VIVO* AND RUMINAL PARAMETERS OF AMAIZE SUPPLEMENTATION *IN VITRO*

Programmed Nutrition Beef Program (Alltech Inc.) is a dietary supplement that contains *Aspergillus oryzae* fermentation extract with α-amylase activity that could reduce the use of feed additives such as monensin and tylosin in conventional feedlot diets. This study examines the changes in rumen fermentation resulting from feeding monensin/tylosin and Programmed Nutrition Beef Finisher. The goal was to determine if these shifts in rumen fermentation contribute to the Programmed Nutrition response observed *in vivo.* This study also analyzes how different levels of α-amylase (Amaize) supplementation impacts feedstuff degradation and utilization *in vitro*. *In vivo* experiment used eight steers in a replicated 4 x 4 Latin square design, and measured heat production, ruminal pH, and ruminal volatile fatty acid (VFA) concentrations for 48 hours. The *in vivo* study measured by urine and fecal output; dietary digestibility, nitrogen balance, ^{15}N-glycine protein turnover, and energy balance. *In vitro* experiment used rumen fluid from three cannulated steers to measure *in vitro* gas production, rate of degradation, and starch degraded for different feedstuffs with or without Amaize. *In vivo* study determined that inclusion of Programmed Nutrition did not increase average daily gain, ruminal pH, ruminal VFA production, nitrogen digestibility, or methane production. Feeding Programmed Nutrition with monensin and tylosin can decrease acid detergent fiber (ADF) digestibility. *In vitro* study determined that *in vitro* gas production after 24-h increases when dry rolled barley is incubated with Amaize. Rate of degradation increases when dry rolled corn, corn steam-flaked at 26 lb/bu, retrograde steam-flaked corn, and steam-flaked barley are incubated with Amaize. Starch degraded after 2-h incubation increases when steam-flaked corn and retrograded steam-flaked corn are incubated with Amaize.

KEYWORDS: Programmed Nutrition, monensin, tylosin, α-amylase, Amaize

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January, 10th 2022
Date

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FREQUENTLY USED ABBREVIATIONS

ADF	Acid Detergent Fiber
ADG	Average Daily Gain
AF	As-Fed
ATP	Adenosine triphosphate
BW	Body Weight
CH_4	Methane
CO_2	Carbon Dioxide
CP	Crude Protein
d	Day
DE	Digestible Energy
DM	Dry Matter
DMI	Dry Matter Intake
DRB	Dry Rolled Barley
DRC	Dry Rolled Corn
h	Hour
HMC	High Moiusture Corn
HP	Heat Production
IU	International Units
M	Monensin
min	Minute
ME	Metabolizable Energy
MT	Conventional trace mineral supplement + Monensin + Tylosin
N	Nitrogen
NDF	Neutral Detergent Fiber
O_2	Oxygen
OM	Organic Matter
OMI	Organic Matter Digestibility
PN	Programmed Nutriton Beef Finisher
PNMT	Programmed Nutrition Beef Finisher + Monensin + Tylosin

RE	Retained Energy
Retro	Retrograde steam-flaked corn
SEM	Standard Error of the Mean
SFB	Steam-Flaked Barley
SFC	Steam-Flaked Corn
T	Tylosin
VFA	Volatile Fatty Acid

CHAPTER 1.

Introduction

Beef cattle usually consume forage-based diets prior to entry in the feedlot, where they are finished on high-grain or high-concentrate diets. These high-grain diets are offered to cattle because of their high energy content. The transition from a forage-based diet to a grain-based diet can create metabolic disorders that cause negative impacts on animal health and performance. One of the metabolic disorders associated with grain-based diets is ruminal acidosis, driven by rapid hydrolysis of starch by rumen microorganisms. The consumption of rapidly fermentable carbohydrates can cause an accumulation of volatile fatty acids and lactate in the rumen, depressing rumen pH and inducing rumen acidosis.

Monensin and tylosin are commonly fed in feedlots to prevent these negative impacts that can occur during the transition phase. Monensin is an ionophore antibiotic that can prevent decreases in rumen pH by reducing lactic acid production and increasing propionate production in the rumen. Tylosin is a macrolide antibiotic that has been shown to reduce liver abscess by inhibiting *Fusobacterium necrophorum* growth, the primary microorganism associated with liver abscess when animals experience rumen acidosis.

Programmed Nutrition (Beef Finisher; Alltech Inc.) is a blend of proteinated trace minerals, *Aspergillus oryzae* fermentation extract, *Lactobacillus acidophilus,* and *Enterococcus faecium* fermentation products. Previous studies have shown that Programmed Nutrition can promote animal feedlot performance similar to conventional

feedlot feed additive systems. This product aims to improve animal health and productivity without using ionophore and antibiotic feed additives such as monensin and tylosin.

Aspergillus oryzae fermentation extract with α-amylase activity (Amaize) is one of the Programmed Nutrition Beef Finisher components. The effects *Aspergillus oryzae* on the rumen can vary greatly. Some studies observed differences in rumen variables, while others reported no changes. There is no clear understanding of the mechanisms of action of *Aspergillus oryzae* fermentation extract with α-amylase activity and how it affects feedstuffs in the rumen.

The goal of this dissertation was to understand how Programmed Nutrition Beef Finisher compares to a traditional feedlot program nutrition (monensin + tylosin) when analyzing ruminal and whole-body variables *in vivo*. Since *Aspergillus oryzae* fermentation extract with α-amylase activity (Amaize) is a Programmed Nutrition Beef Finisher component, we evaluated its effects on *in vitro* activity when incubated with different feedstuffs. This would allow a better understanding of how Amaize impacts ruminal fermentation and starch degradation of different grain types.

CHAPTER 2.

Literature Review

2.1. Metabolism of carbohydrates by rumen microorganisms

Microorganisms in the rumen can digest structural and non-structural carbohydrates present in the feed (Schofield et al., 1994; Dai et al., 2015). Via anaerobic fermentation, rumen microbes utilize carbohydrates to generate energy for maintenance and growth (Baldwin and Allison, 1983). Microbial enzymes break down polysaccharides such as cellulose, hemicellulose, pectin, and starch to monosaccharides such as glucose, fructose, and xylose. Lignin is not degraded during anaerobic fermentation since it is a hydrophobic polymer with aromatic phenolic residues that require oxygen for its degradation (White et al., 2014). Cellulose is a straight-chain polymer composed of repeating glucose units linked via β-1,4-glycoside bonds (Schwarz, 2001). This linear chain allows the formation of hydrogen bonds within the polymer, which gives structural rigidity to the molecule (Bayer et al., 1998). Hemicellulose is also a linear chain formed by xylose chains with arabinose, galactose, and uronic acids. Pectins are linear chains composed of galacturonic acid with other sugars within or as side chains (Schwarz, 2001). Starch is composed of amylose and amylopectin, where the first is a mostly linear α-1,4-glucan, and the second is an α-1,4-glucan with α-1,6-linkages at the branch point (Bastioli et al., 2014).

Rumen microorganisms attach to feed particles via specific and nonspecific adhesion mechanisms (Pell and Schofield, 1993). Generalized low-affinity forces such as van der Waals forces are involved in nonspecific adhesion, which can be reversible or irreversible governed by Brownian motion (Staat and Peyton, 1984; Busscher and Weerkamp, 1987). Specific adhesion occurs via ligands or lectin-like adhesions on the bacterial cell surface and receptors on the feed particle (Gibbons, 1984). Bacteria can present different adhesions, allowing attachment to other surface receptors (Van der Mei et al., 1987). The affinity of bacterial starch surface receptors decreases when starch is treated with mixtures of alcohol and HCl, which indicates that cereal grains treated with similar compounds can have lower starch digestion rates due to lower bacterial attachment (McAllister et al., 1994).

Starch-degrading rumen bacteria produce enzymes with α-amylase activity that generates specific groups of oligosaccharides (Cotta, 1988). Enzymes of starch-degrading bacteria can be located in the bacterial periplasm or cytoplasm, where starch is transported across the outer membrane to these sites via cell-surface proteins (Anderson and Salyers, 1989a, 1989b). Starch-degrading bacteria produce enzymes capable of starch digestion but do not produce the variety of enzymes required to digest the entire cereal grain, so they must rely on other bacterial species enzyme activities to gain access to starch granules (Cotta, 1988; McAllister et al., 1994). Bacteria and fungi access the readily digestible inner tissues via vulnerable regions of the plant tissues such as the stomata and damaged areas (McAllister et al., 1994).

Fungi can reach regions of the plant that bacteria can’t due to their ability to penetrate the plant tissue and generate additional sites for bacterial attachment and produce

extracellular enzymes that degrade the plant cell wall (Pell and Schofield, 1993). Fungi zoospores rapidly attach to feed particles, encyst, introduce their hyphae into the plant tissue and further grow rhizoids into the plant cell wall, which weakens the plant tissue and facilitates bacterial attachment (Bauchop, 1981; Akin, 1986). Rumen conditions affect the anaerobic fungi population. Zoospore production and attachment to feed particles decreases when rumen pH is below 5.5, and reduction of acetate:propionate ratio causes a decline in the fungal population (Grenet et al., 1989).

Microorganisms metabolize pentoses by converting three pentoses into two hexoses and one triose, via transaldolase and transketolase (Wallnofer et al., 1966; Baldwin and Allison, 1983). The Embden-Meyerhof pathway is the primary pathway utilized by microbes to generate energy (ATP) during hexose fermentation. In glycolysis, hexoses can be converted to 2 pyruvate molecules via H_2-forming/electron-donating reactions, utilizing 2 NADH and 2 ADP, and generating 2 NAD^+ and 2 ATP molecules (Baldwin and Allison, 1983). Carbohydrate anaerobic fermentation generates microbial protein and metabolites that can be utilized by the animal host (Ceconi et al., 2015). Volatile fatty acids (VFA), lactate, methane (CH_4), CO_2, and H^+ are byproducts of the microbial synthesis of adenosine triphosphate (ATP) (Thauer et al., 1977). Formation of byproducts occurs to maintain the hydrogen balance in the system via H_2-consuming/Electron-accepting and H_2-forming/Electron-donating partial reactions (Baldwin and Allison, 1983).

Volatile fatty acids (VFA), also called short-chain fatty acids (SCFA), are weak acids ($pKa \leq 4.8$) that can exist in the dissociated anion or ionized state. In the rumen, these acids include formate (C1), acetate (C2), propionate (C3), butyrate (C4), valerate (C5), isobutyrate (C4), isovalerate (C5), and 2-methylbutyrate (C5) (Bergman, 1990). Acetate is

produced when pyruvate is converted to acetyl-CoA, which has its CoA group replaced by a phosphate (Pi) forming acetyl-P that can be converted to acetate by donating its phosphate group to ADP to generate ATP (Thauer et al., 1977). Propionate can be produced by succinate or acrylate pathways. In the succinate pathway, pyruvate is converted to oxaloacetate using one ATP, which is reduced to malate that is later converted to fumarate, and succinate is formed from fumarate by oxidizing NADH. Succinate can be converted to succinyl-CoA by utilizing the CoA group from propionyl-CoA, releasing propionate. The last cycle can be repeated since succinyl-CoA can be rearranged to form methylmalonyl-CoA, which can be converted to propionyl-CoA and reutilized in the previous reaction. The acrylate pathway occurs when pyruvate is reduced to lactate, a mechanism used to remove intracellular H^+ and control intracellular pH (Baldwin and Allison, 1983). To regenerate NAD^+ used in glycolysis, pyruvate can be converted to lactate by microbial lactate dehydrogenase via H_2-consuming/electron-accepting process where pyruvate accepts electrons from NADH. Once lactate is formed, it can be used to produce lactyl-CoA, using an acetyl-CoA and releasing an acetate. Lactyl-CoA is dehydrated, forming acryloyl-CoA that is reduced to propionyl-CoA, that can be used with an acetate to produce propionate. Butyrate (VFA) is generated by converting two molecules of acetyl-CoA to acetoacetyl-CoA, which is reduced to β-hydroxybutyryl-CoA, dehydrated to crotonyl-CoA and reduced to butyryl-CoA. The CoA group is then replaced by a phosphate (Pi), forming butyryl-P, which is dephosphorylated into butyrate, releasing an ATP (Thauer et al., 1977). Lactate is an organic acid (pK = 3.86) that can be synthesized from pyruvate, as mentioned previously. Lactate in the rumen can be converted to propionate via acrylate pathway, to

acetate and to butyrate by being oxidized back to pyruvate (Johns, 1951a, 1952; Jayasuriyal and Hungate, 1959; Satter and Esdale, 1968; Russell, 1998; Mills et al., 2014).

Gaseous H_2 is produced during the oxidation of NADH to NAD^+ by enzymes present in the bacterial membrane. Synthesis of two molecules of pyruvate from glucose via Embden-Meyerhof pathway yields two H_2, and production of one molecule of acetate from pyruvate yields one H_2 and one CO_2. Other reactions serve as H_2 sinks, where conversion of one pyruvate to one propionate consumes two H_2. Conversion of two acetyl-CoA to one butyrate consumes two H_2. Methanogenic bacteria can utilize four H_2 and one CO_2 to synthesize one methane (CH_4) and two H_2O (Hino and Russell, 1985; Moss et al., 2000; White et al., 2014).

It has long been desired of researchers to acquire control of rumen microbial metabolism in order to optimize metabolite utilization by the animal and improve economic feedback. Ruminal fermentation modifiers such as monensin and tylosin have been used to manipulate rumen function for many years. Some effects of monensin on rumen fermentation pattern are well established, but its complete mode of action is not yet clearly understood. Monensin can affect rumen fermentation patterns by inhibiting the growth of gram-positive organisms, increasing the molar proportion of propionate, and decreasing acetate (Richardson et al., 1976). This effect of monensin on VFA proportions can increase feed efficiency since the microbial synthesis of propionate is more energetically advantageous (Hungate, 1966). The impact of monensin on the rumen microbial population has been studied previously, and its changes on the rumen microbiome can vary. It has been shown that monensin can decrease ruminal protozoal numbers (Guan et al., 2006), while other studies did not detect changes in the protozoal population (Dinius et al., 1976).

These differences could be due to the levels of monensin supplementation and the capacity of protozoal populations to adapt to the ionophore. The effects of monensin on ruminal bacterial have also been reported, where monensin has been found to inhibit formate-producing, hydrogen-producing, and lactic acid-producing bacteria, while stimulating succinate-producing and propionate-producing bacteria (Chen and Wolin, 1979; Dennis et al., 1981). Dennis et al. (1981) reported that monensin only inhibited four out of seven different lactic-acid producing bacteria but did not affect three strains of bacteria that convert lactate to propionate. These findings suggest that monensin may decrease lactate synthesis in the rumen without affecting lactate to propionate metabolization.

Direct-fed microbials also have been used to manipulate rumen microbial fermentation patterns. Bacterial strains from the genera *Propionibacterium* and *Lactobacillus* have the potential to reduce CH_4 synthesis by converting lactate to propionate in the rumen (Jeyanathan et al., 2016; Vyas et al., 2016). These improvements in CH_4 might not be observed when animals consume grain-based diets once propionate levels are already high with increased rapidly fermented carbohydrate intake (Vyas et al., 2014). The reduction in CH_4 production is not always accompanied by changes in propionate synthesis (Vyas et al., 2014; Jeyanathan et al., 2016), which indicates that H^+ availability is not the only factor in CH_4 synthesis. Direct-fed microbials have the potential capacity to modify methanogenic bacterial and archaeal profiles (Janssen and Kirs, 2008; Poulsen et al., 2013). The capacity of direct-fed microbials to modify the rumen microbial environment is correlated to the extent of their ruminal survival and activity (McAllister et al., 2011; Jeyanathan et al., 2016). The reason why direct-fed microbials are usually fed on a daily basis is that they have difficulties integrating permanently with the rumen microbial

community, even if the direct-fed microbials are originated from rumen sources (McAllister et al., 2011). One of the factors influencing the integration of direct-fed microbials in the rumen is quorum sensing, the release of small signal molecules as a method of communication between bacterial cells (Mitsumori et al., 2003). It is not known currently if direct-fed microbials use the same set of signal molecules to communicate as the microorganisms present in the rumen.

Direct-fed microbials fed to ruminants often combine lactic acid-producers and lactic acid-utilizers. The rationale for this system is that lactic acid synthesis in the rumen promotes the growth of lactic-acid utilizing bacteria, generally converting lactic acid to propionate (McAllister et al., 2011). The additional population of lactic-acid utilizers fed via direct-fed microbials would ensure all lactic acid produced in the rumen is rapidly metabolized. This would avoid possible metabolic disorders caused by lactic acid accumulation in the rumen (Owens et al., 1998; Krause and Oetzel, 2006). A decrease in ruminal lactate does not always occur with the supplementation of direct-fed microbials. Kenney et al. (2015a) reported no differences in the ruminal rate of lactate disappearance when supplementing a direct-fed microbial containing primarily *Lactobacillus acidophilus* and *Enterococcus faecium* indicating that potentially not all species of lactate-producing direct-fed microbials alter ruminal dynamics in the same fashion.

2.2. Ruminant animal metabolism of carbohydrates and fermentation products

The majority of carbohydrates in the feed ingested by the animal are fermented in the rumen, generating microbial mass and other byproducts as described previously. Most

animal energy requirements are met via absorption of VFA produced during anaerobic fermentation in the rumen (Hungate, 1966; Baird et al., 1980). Volatile fatty acids are mainly absorbed via passive diffusion by the rumen epithelium (> 90%), and a small portion can escape the rumen and be absorbed in the small intestine (Harfoot, 1978). Rumen epithelial cells metabolize mostly butyrate, to some extent propionate, and negligent amounts of acetate (Pennington, 1951; Bergman, 1990). All three VFAs can be oxidized via the Krebs Cycle in the mitochondria of rumen epithelial cells. A small portion of propionate can also be converted to lactate (Weekes, 1974) and alanine (Bergman, 1990). Butyrate can also be converted to ketone bodies (β-hydroxybutyrate and acetoacetate) in the mitochondria of the rumen epithelial cell (Pennington, 1951; Leighton et al., 1983).

The portion of products that were not metabolized by the rumen epithelial cells are transported to the bloodstream, reaching the liver via the hepatic portal vein (Baird et al., 1980; Harmon et al., 1988; Bergman, 1990; Kristensen and Harmon, 2004). Acetate is usually not metabolized to a great extent by hepatocytes. It is utilized as an energy source mostly by the peripheral tissues and some by the portal-drained viscera (gastrointestinal tract, pancreas, spleen, and associated omental and mesenteric fat) (Holdsworth et al., 1964; Bergman and Wolff, 1971; Demigne et al., 1986; Seal and Reynolds, 1993). Propionate is mainly converted to glucose by the hepatocytes and released into the bloodstream to other tissues (Bergman, 1990; Harmon et al., 1993; Reynolds, 2002). Butyrate is converted to β-hydroxybutyrate, similarly to the rumen epithelial cells. The ketone bodies produced by the rumen epithelial cells also reach the hepatic portal vein, where acetoacetate is converted to β-hydroxybutyrate by the hepatocytes. The β-

hydroxybutyrate present in the liver is then transported to the hepatic vein, where it can reach peripheral tissues (Bergman, 1970; Demigne et al., 1986; Reynolds, 2002).

The gases CO_2 and CH_4 are released via eructation, but a small portion of CH_4 can be absorbed by the rumen epithelium, transported by the bloodstream to the lungs, where it is expired into the environment (Johnson and Johnson, 1995; Freetly and Brown-Brandl, 2013). The fraction of carbohydrates that did not ferment in the rumen can flow into the small intestine, where it is hydrolyzed by pancreatic and intestinal enzymes to produce glucose that is absorbed (Owens et al., 1986; Shirazi-Beechey et al., 1991a).

2.3. Metabolic impacts of grain-based diets in cattle

High-grain diets promote a greater concentration of glucose in the rumen due to the cleavage of starch by microorganisms. This glucose accumulation can lead to the rapid growth of microbial populations, which promotes VFA and lactate production (Owens et al., 1998; Sutton et al., 2003; Penner et al., 2009). An increase in starch and other rapidly fermentable carbohydrates consumption can lead to the accumulation of these organic acids, which can depress rumen pH (Owens et al., 1998; Vasconcelos and Galyean, 2008). Lactate and VFA absorption through the rumen wall are higher when protonated and rumen pH is low (Bergman, 1990). Lactate is a stronger acid and less protonated than VFA, leading to an easier accumulation of this metabolite in the rumen (Mills et al., 2014).

Ruminal acidosis can be classified as subacute (SARA) when rumen pH is below 5.5 for a minimum of 3 hours. Acute acidosis is characterized by a rumen pH below 5.0 (Owens et al., 1998; Krause and Oetzel, 2006). Subacute acidosis is characterized by the

accumulation of VFA in the rumen, while lactate accumulation is the main factor driving acute acidosis (Therion et al., 1982; Krause and Oetzel, 2006; Nagaraja and Titgemeyer, 2006). Lactate-utilizing microorganisms are sensitive to low pH, but lactate-producers are not, promoting lactate accumulation in the rumen when pH is low (Owens et al., 1998). For this reason, during subacute acidosis, lactate-utilizing bacteria are actively metabolizing lactate which avoids an accumulation of this acid (Goad et al., 1998). But as pH drops even further, the growth of lactate-utilizers is inhibited, causing lactate accumulation (Krause and Oetzel, 2006; Nagaraja and Titgemeyer, 2006).

When the metabolite concentration in the rumen increases rumen, osmolarity increases, moving water from the blood through the rumen wall to the rumen lumen. This water movement can cause damage to the rumen epithelium (rumenitis), which can later be a port of entry for microorganisms that cause rumen and liver abscesses (Nagaraja and Chengappa, 1998; Steele et al., 2011). The repair of the damaged rumen wall can cause thickening of the tissue (parakeratosis) which can reduce VFA absorption, ultimately causing its accumulation in the rumen lumen (Harris, 1965).

2.4. Monensin

Monensin is a monovalent carboxylic polyether ionophore antibiotic produced by *Streptomyces cinnamonensis* bacteria (Agtarap et al., 1967; Day et al., 1973; Pressman and deGuzman, 1976; Butaye et al., 2003). This ionophore structure comprises a hydrophobic/lipophilic alkyl backbone and an internal oxygen-rich polar cavity that can bind to metal ions while shielding their charge. This structure allows the antibiotic to

transport alkali metal cations $Na^+ > K^+ > Rb^+ > Li^+ > Cs^+$ (affinity order) across biological membranes, disrupting the ion transport system and permeability of prokaryotes and eukaryotes cells. Factors affecting cation affinity to monensin are cation size, ligand distribution, and ionophore flexibility. In normal conditions, the concentration of Na^+ is high in the extracellular environment, and of K^+ is high intracellularly. Monensin can penetrate the cell membrane, disrupt this cation balance, and cause cell death (Lutz et al., 1971; Pressman and deGuzman, 1976; Pangborn et al., 1987; Dorkov et al., 2008; Kevin II et al., 2009). The ionic gradient imbalance triggers Na^+/K^+-ATPase that uses ATP to pump Na^+ from the cytoplasm. The energy expenditure required to maintain ion balance within the cell leads to inhibition in cell growth and later cell death (Ferguson et al., 2006; Wang et al., 2006; Newbold et al., 2013). Due to its high molecular weight, monensin can penetrate the membrane of gram-positive but usually not gram-negative bacteria. The outer membrane present in gram-negative bacteria is impermeable to hydrophobic/lipophilic compounds, preventing monensin from entering the cell (Alleaume and Hicke, 1972; Guyot et al., 1993; Kevin II et al., 2009).

Monensin is a sodium salt can be used as a dietary supplement for cattle to increase feed efficiency, decrease fasting heat production, improve dry matter digestibility and nitrogen balance. Ruminal parameters are also enhanced with monensin, where the reduction of lactic acid production aids in maintaining a greater rumen pH (Steen et al., 1978; Goodrich et al., 1984; Duffield et al., 2012). Monensin also increases the ruminal molar percentage of propionic acid while decreasing acetic and butyric acid. Which leads to a higher glucose supply from gluconeogenesis of propionate in the liver (Richardson et al., 1976; Prange et al., 1978; Lomax et al., 1979; Baird et al., 1980).

Monensin can be used to prevent the negative impacts of diets high in rapidly fermentable carbohydrates in ruminants. This antibiotic can allow a higher intake of rapidly fermented carbohydrates without causing metabolic disturbances (Burrin and Britton, 1986; Burrin et al., 1988). Monensin and tylosin can aid ruminal adaptation during the transition from a low grain to a high grain diet, reducing daily intake variances and improving animal performance (Burrin et al., 1988). When monensin and tylosin are fed to feedlot cattle consuming grain-based diets, it can improve gain:feed by reducing dry matter intake (Potter et al., 1985; Stock et al., 1995). It has been reported that when monensin is fed to feedlot cattle, it can cause 1.6% faster weight gain, increase ribeye area by 0.61%, reduce animal energy requirements, improve dietary energy values and improve apparent digestibility by 6.5% (Goodrich et al., 1984).

2.5. Tylosin

Tylosin is a macrolide antibiotic formed by a 16-member lactone ring with one monosaccharide formed by a mycinose residue at position 14 and two disaccharides formed by mycaminose and mycarose residues at positions 5 and 16. This macrolide also contains a reactive acetaldehyde group at position 6 (Sumbatyan et al., 2003; Aronson, 2016; Papich, 2016). Tylosin affects gram-positive and some gram-negative bacteria, causing cell death by inhibiting bacterial protein synthesis. It causes mechanical obstruction to the nascent polypeptide chain. The macrolide binds approximately 20Å from the peptidyl transferase center in the 50S bacterial ribosome, placing its lactone ring orthogonally to the ribosomal tunnel, where nascent polypeptide chains emerge during protein synthesis

(Schlunzen et al., 2001; Hansen et al., 2002; Sumbatyan et al., 2003). The wide use of Tylosin raises concerns due to acquired resistance observed in potential human pathogens such as Enterococcus faecium (Aronson, 2016).

Liver abscesses can occur when the animal is experiencing rumen acidosis. It is characterized as a pus-filled capsule with necrotic centers containing degenerated hepatocytes and leukocytes. *Fusobacterium necrophorum* is the primary ruminal microorganism associated with liver abscesses in cattle. This gram-negative bacteria is resistant to monensin and can gain access to the bloodstream and the liver when the rumen epithelial wall is damaged during acidosis (Tan et al., 1996; Narayanan et al., 1997; Nagaraja and Chengappa, 1998; Nagaraja et al., 2005; Steele et al., 2011; Amachawadi et al., 2017). Tylosin can be included in the diet of ruminants to reduce the growth of *Fusobacterium necrophorum* in the rumen and liver (Lechtenberg et al., 1998; Nagaraja and Chengappa, 1998; Nagaraja et al., 1999; Amachawadi et al., 2017).

2.6. *Lactobacillus acidophilus* and *Enterococcus faecium*

Gram-positive lactate-producing bacteria *Lactobacillus acidophilus* and *Enterococcus faecium* are commonly supplemented as antibiotic alternatives to ruminants. These probiotics can be used to assist microbial adaptation to the presence of lactic acid in the rumen. Low and sustained production of lactic acid by lactate-producing bacteria can stimulate the growth of lactic acid-utilizing bacteria, which would stabilize rumen pH when an accumulation of lactic acid occurs in later stages of acidosis (Owens et al., 1998;

Ghorbani et al., 2002; Nocek et al., 2002; Callaway and Ricke, 2012). Higher rumen pH can lead to higher DMI and feed digestibility (Nocek et al., 2003; Nocek and Kautz, 2006).

Outcomes can vary when supplementing probiotics to cattle due to differences in diet composition, animal characteristics, and probiotic dosage. Vasconcelos et al. (2008) observed in steers a 2 to 3% increase in feed efficiency when supplementing gram-positive lactate-utilizer *Propionibacterium freudenreichii* (1×10^9 cfu/steer/d) with *Lactobacillus acidophilus* at low (1×10^7 cfu/steer/d) and high (1×10^9 cfu/steer/d), but not at medium (1×10^8 cfu/steer/d) doses. Timmerman et al. (2005) observed an increase in ADG and feed efficiency when supplementing calves with a calf-specific multistrain probiotic containing *Lactobacillus acidophilus* and *Enterococcus faecium*. Dawson et al. (1990) observed an increase in rumen pH when supplementing steers with yeast *Saccharomyces cerevisiae* (2.04×10^9 cfu/g), *Lactobacillus acidophilus* (1.31×10^8 cfu/g), and *Enterococcus faecium* (7.5×10^5 cfu/g). Kenney et al. (2015a) did not observe differences in DMI, ADG, growth efficiency, DM digestibility, nitrogen digestibility, rumen pH, and rumen lactate disappearance when supplementing steers with *Lactobacillus acidophilus*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Lactobacillus brevis,* and *Lactobacillus plantarum* (1×10^9 cfu/steer/d). However, Kenney et al. (2015b) demonstrated that degradable intake protein content in the animal diet influences the response to probiotic supplementation. In Kenney et al. (2015b) study, animals receiving a mixed bacterial culture consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium* (1×10^9 cfu/steer/d) presented a cubic increase in ADG when consuming degradable intake protein above the calculated animal requirement. Their data suggests that greater degradable protein content in the diet might be required to maximize the benefits of probiotic supplementation.

2.7. *Aspergillus oryzae* fermentation extract with α-amylase activity

The yeast *Aspergillus oryzae* can produce starch-degrading enzymes (amylase and amyloglucosidase) and proteolytic enzymes (Fogarty and Kelly, 1979; Boing, 1983). Adding of this yeast to the rumen environment can modify the microorganism population and fermentation pattern (Newbold, 1996). Yeast can remove oxygen from the rumen preventing toxicity to anaerobe microorganisms (Newbold et al., 1993, 1996). Lactic acid utilization can be improved with yeast supplementation since many anaerobic bacteria are lactate-utilizers (Nisbet and Martin, 1990, 1991; Williams et al., 1991; Martin and Nisbet, 1992). A drop in rumen pH can be avoided by preventing lactate accumulation in the rumen, leading to enhanced growth of pH-sensitive microorganisms (Owens et al., 1998).

Results regarding changes in ruminal fermentation with *Aspergillus oryzae* supplementation can vary. An increase in total VFA concentrations has been observed (Nisbet and Martin, 1991; Varel and Kreikemeier, 1994b) and increased molar proportions of acetate and propionate (Harrison et al., 1988; Nisbet and Martin, 1991). In contrast, other studies obtained no differences in VFA (Carro et al., 1992; Caton et al., 1993; Fiems et al., 1993; Higginbotham et al., 1994). The reason of why theses discrepancies in response to VFA metabolism occurs are not yet well understood. *Aspergillus oryzae* can improve cellulose digestion by stimulating increase in concentration of fibrolytic bacteria *in vivo* (Wiedmeier et al., 1987; Harrison et al., 1988; Newbold et al., 1992; Newbold, 1996) and *in vitro* (Dawson et al., 1990; Newbold et al., 1991).

Aspergillus oryzae can supply limiting amino acids required for the growth of specific microbial populations, improving microbial protein synthesis (Erasmus et al.,

1992; Newbold, 1996). Other studies have found no difference in ruminal microbial populations with *Aspergillus oryzae* supplementation (Oellermann et al., 1990; Varel and Kreikemeier, 1994b), indicating that this effect can vary depending on feed ingredients used in the animal diet. Overall, an increase in microbial populations in the rumen can increase feed digestibility which can increase passage rate, leading to a higher intake and gain (Newbold, 1996). Increases in ADG have been observed when supplementing steers with *Aspergillus oryzae* (Allison and McCraw, 1989). Calves supplemented with this yeast were weaned one week earlier and presented higher numbers of ruminal bacteria and rumen fermentation activity (Beharka et al., 1991).

2.8. Vitamins and minerals

Vitamin and mineral supplements are used in the diet of cattle to meet the animal and microbial requirements. Vitamins A, D, E, and K are classified as lipid-soluble. Choline, biotin, folic acid, niacin, riboflavin, thiamin, vitamin C, B_{12}, B_5, and B_6 are classified as water-soluble. Minerals calcium (Ca), phosphorous (P), magnesium (Mg), potassium (K), sodium (Na), chloride (Cl), and sulfur (S) are classified as macrominerals and are required by beef cattle in grams/day. Meanwhile, cobalt (Co), copper (Cu), iodine (I), manganese (Mn), iron (Fe), molybdenum (Mo), zinc (Zn), and selenium (Se) are classified as microminerals or trace minerals, and their requirements are in milligrams or micrograms/day. Supplementation of some of these vitamins and minerals is often necessary to meet the requirements due to being insufficient in feedstuffs (NRC, 2016).

Vitamin C is not required in the diet of ruminants since it can be synthesized by the animal. Likewise, vitamin K and water-soluble vitamins can be synthesized by ruminal bacteria and absorbed by the animal in the small intestine. Vitamin E supplementation for nonstressed mature beef cattle is likely not necessary as its requirements are low and often met through feedstuffs in the diet (NRC, 2016). Vitamin A is stored in the liver (Hayes et al., 1967), is involved in the maintenance of epithelial tissues (Perry and Cecava, 1995), synthesis of retinaldehyde required for dim light vision (Moore, 1939, 1941; Byers et al., 1956), and it is essential for bone development, growth and reproduction (NRC, 2016). Vitamin A toxicity in ruminants is uncommon since microorganisms can degrade it in the rumen (Rode et al., 1990).

Vitamin D exists in two forms, ergocalciferol (vitamin D_2) derived from ergosterol (plant steroid) and cholecalciferol (vitamin D_3) derived from precursor 7-dehydrocholesterol present in animal tissues and products. Mammals store a limited amount of vitamin D in tissues (NRC, 2016). Cattle can synthesize vitamin D from 7-dehydrocholesterol present in the skin with exposure to sunlight. Supplementation of this vitamin is necessary when cattle populate an area with low levels of sunlight, such as closed confinement. Vitamin D is required to absorb calcium and phosphorous from the gut (DeLuca, 1979), bone mineralization, and calcium mobilization from the bone. A limited amount of this vitamin can be stored in the liver, kidney, and lungs. Vitamin D toxicity can occur, causing decreases in DMI, calcification of soft tissues, and bone demineralization (NRC, 2016).

There is a limited amount of information about how monensin and direct-fed microbials affect vitamin metabolism in ruminants. Agustinho et al. (2021) reported lower

somatic cell count when multiparous Holstein dairy cows were fed monensin and vitamin A, likely due to the capacity of monensin to improve immune function due to better utilization of endogenous energy sources. High-grain diets can cause a decrease in the synthesis of true vitamin B_{12} in the rumen and increase the synthesis of vitamin B_{12} analogs, where these analogs cannot act as enzyme cofactors (Sutton and Elliott, 1972). Monensin supplementation in high-grain diets could decrease liver vitamin B_{12} status, where true vitamin B_{12} synthesis is affected by ruminal propionate synthesis (Sutton and Elliott, 1972; Daugherty et al., 1986). Daugherty et al. (1986) did not observe improvements in animal performance when supplementing vitamin B_{12} and monensin to lambs but reported a decrease in fumarate and malate production by 50%. They hypothesized that monensin and vitamin B_{12} on fumarate and malate production was due to monensin inhibitory effect on enzymes of the Krebs cycle. Wiedmeier et al. (1987) observed a 27% increase in the ruminal cellulolytic bacteria when supplementing *Aspergillus oryzae* fermentation extract. They hypothesized this growth occurred due to stimulatory factors produced by *Aspergillus oryzae* such as B vitamins.

Calcium is primarily present in the bones and teeth (98%), with a small amount (2%) present in soft tissues and extracellular fluids. Calcium is absorbed mainly from the duodenum and jejunum via passive diffusion and active transport (McDowell, 2003). Calcium is required for membrane permeability, muscle contraction, nerve impulse transmission, hormone secretion, enzyme activity, blood clotting, and cardiac regulation (NRC, 2016). Phosphorous is found in soft tissue (20%), bone and teeth (80%). It is part of the structure of DNA and RNA, ATP/ADP/AMP, phospholipids, nucleoproteins,

riboflavin-phosphate enzyme complex, diphosphothiamin, pyridoxal phosphate, and phosphoric acid esters of carbohydrates (Perry and Cecava, 1995; NRC, 2016).

Magnesium is a macromineral present in the bones (65-70%), muscle (15%), other soft tissues (15%), and extracellular fluid (1%) (Mayland, 1988). It is primarily absorbed from the rumen via an active sodium-like process (Grace et al., 1974; Martens and Rayssiguier, 1980; Greene et al., 1983; Greene et al., 1989). Magnesium is required to activate many enzymes as it is part of the Mg-ATP complex, and it maintains the electrical potential of membranes of muscle and nerve cells (Perry and Cecava, 1995; NRC, 2016).

Potassium is the cation in higher concentration intracellularly. It is involved in some enzymatic reactions, acid-base regulation, osmolarity, nerve impulse transmission and muscle contraction. This mineral is absorbed from the rumen, omasum, and intestine (NRC, 2016). The major minerals in the extracellular fluid of the cells are the cation sodium and the anion chlorine (Cl). They are involved in regulating water balance, acid-base balance, and osmotic pressure within the cell. Sodium is absorbed from the small intestine, and it is required for glucose and amino acid transport, nerve impulse transmission, and muscle contraction. Chlorine is absorbed primarily in the intestine and is present in the hydrochloric acid portion of the gastric juice (Perry and Cecava, 1995; NRC, 2016).

Sulfur is a macromineral in organic compounds such as methionine, cysteine, cystine (amino acids), thiamin and biotin (vitamin B). Ruminal microorganisms can utilize inorganic sulfur to synthesize all animal-required organic sulfur-containing compounds. Sulfur can be toxic in high concentrations (> 0.4% of the diet), causing a reduction in dry

matter intake (DMI), slow growth, diarrhea, muscle twitching, and even death (Perry and Cecava, 1995; NRC, 2016).

Cobalt is utilized by bacteria to synthesize vitamin B_{12}, which is required by vitamin B_{12}-dependent enzymes methylmalonyl CoA mutase and 5-methyltetrahydrofolate homocysteine methyltransferase (Smith, 1987). Metalloenzymes ribonucleotide reductases which reduce ribonucleoside diphosphates (NDP) and triphosphate (NTP) during DNA synthesis and requires the transition metals Co and Fe (Mulliez and Fontecave, 1997). Cobalt deficiency can cause weight loss, fatty degeneration of the liver, anemia, and impaired ability to fight infections (Smith, 1987; MacPherson et al., 1988). Cobalt toxicity is unlikely to occur since cattle can tolerate up to 100 times its requirement (NRC, 2016). Copper acts as a component of several enzymes such as cytochrome oxidase and superoxide dismutase (McDowell, 2003). Its requirement varies depending on the concentration of antagonistic Mo and S in the diet. Thiomolybdates are formed in the rumen via molybdate and sulfide interactions (Suttle, 1991). These compounds can form insoluble complexes with Cu. which affects the absorption and metabolism of this mineral (Gooneratne et al., 1989). Iodine is mostly present in the thyroid gland (70-80%) as inorganic iodine, a constituent of the thyroid hormones thyroxine (T4) and triiodothyronine (T3), and other iodinated compounds (Mertz, 1986). Iodine is mostly absorbed in the rumen (70-80%), also being absorbed in the small and large intestine. The abomasum is a site of endogenous secretion (Miller et al., 1973; Miller et al., 1974; Miller et al., 1988).

Manganese functions as both a constituent of metalloenzymes and as an enzyme activator (McDowell, 2003). It is a component of the enzymes arginase, pyruvate carboxylase, and superoxide dismutase (Hurley and Keen, 1987). Manganese is also

required for the activation of many enzymes such as kinases, decarboxylases, transferases, and hydrolases (Groppel and Anke, 1971; NRC, 2016). Absorption of Mn is poor and occurs in the small intestine. In cattle, about 1% of the ingested Mn is absorbed (Abrams et al., 1977). In ruminants, Mn deficiency can lead to poor soft tissue and bone growth, muscular weakness, and excess body fat (Thomas, 1970; Hidiroglou, 1980).

Iron is a component of many proteins such as hemoglobin and myoglobin which are involved in oxygen transport and utilization, cytochromes, and Fe-S proteins which participate in the electron transport chain. Iron is also required to activate many mammalian enzymes (McDowell, 2003; NRC, 2016). Iron homeostasis is mainly regulated via absorption in the gastrointestinal tract due to the limited capacity of Fe excretion by animals (McDowell, 2003). Iron availability is highest in the ferrous (Fe^{2+}) state (NRC, 2016). Hydrochloric acid present in the abomasum promotes denaturation of protein-bound Fe, and reduction of ferric (Fe^{3+}) to ferrous state (Fe^{2+}). Cysteine and ascorbic acid present in the feed can also enhance Fe absorption by assisting the reduction to ferrous state (Cook and Reddy, 2001). Hemoglobin contains most of the total body Fe (60%), and the liver is the primary site (60%) of Fe stored in tissues as ferritin protein complex (Beard and Dawson, 1997; McDowell, 2003).

The role of molybdenum in the metabolism is only partially understood, and its requirements for cattle are not yet established (NRC, 2016). Molybdenum can improve microbial metabolism in the rumen, and it is a component of the enzymes xanthine oxidase, sulfite oxidase, and aldehyde oxidase (Underwood, 1977; Mills and Davis, 1987). Copper and sulfur are antagonists to Mo, where interactions in the rumen can result in decreased Mo absorption, limit Cu retention, and alter Mo postabsorptive metabolism (Dick, 1953;

Mills and Davis, 1987). High levels of Mo intake can lead to toxicity in cattle, causing diarrhea, anorexia, stiffness, and changes in hair color (Ward, 1978; Kessler et al., 2012).

Zinc is a component of metalloenzymes and is required to activate some enzymes involved in protein, carbohydrate, lipid, and nucleic acid metabolism (Hambidge et al., 1986; Graham, 1991; McDowell, 2003). Zinc is also required by the immune system for lymphocyte production, antibody response, cytokine synthesis, neutrophil function, T-cell development and function, and others (Fraker et al., 1977; Hambidge et al., 1986; Fraker and King, 1998; Shi et al., 1998). In ruminants, Zn is absorbed in the abomasum and the small intestine (Miller and Cragle, 1965; Arora et al., 1969). Zinc deficiency can compromise the cellular DNA repair mechanism, which can lead to unrepaired DNA damage (Mertz, 1986; McDowell, 2003). Zinc is involved in hormone production, secretion and storage, as well as in the efficiency of receptor response. Its deficiency can affect testosterone, insulin, and adrenal corticosteroid hormones production and secretion (McDowell, 2003). Decreases in DMI, growth, feed efficiency, excessive salivation, listlessness, swollen feet, parakeratotic lesions, impaired wound healing, and alopecia are observed in cattle consuming a Zn deficient diet (NRC, 2016).

Selenium is a metalloid with similar chemical properties to sulfur, and it is mainly absorbed in the duodenum and cecum (Wright and Bell, 1966; McDowell, 2003). Selenometalloenzymes are enzymes that contain Se at the active site and catalyze reactions involved in thyroid hormone metabolism (iodothyronine 5'-deiodinase), redox control, and antioxidant defense systems (glutathione peroxidase) (Mertz, 1986; Arthur et al., 1990; Arthur and Beckett, 1994; Foster and Sumar, 1997; McDowell, 2003). Chronic Se toxicity causes lameness, anorexia, deformed hoofs, liver cirrhosis, loss of tail hair, nephritis, and

emaciation (Rosenfeld and Beath, 1964). Acute Se toxicity can cause diarrhea, ataxia, difficulty breathing, respiratory failure, and death (NRC, 2016).

Monensin supplementation has the potential to affect mineral metabolism in ruminants. Monensin can increase sodium-potassium pump activity due to its strong affinity for Na and K ions (Pressman and deGuzman, 1976; Smith and Rozengurt, 1978). Monensin fed to steers consuming a grain-based diet can increase apparent absorption of Mg, P, Na, and increase Mg and P retention (Starnes et al., 1984). Monensin can also increase apparent absorption of Ca, Mg, K, and P in steers consuming forage-based diets (Spears et al., 1989). These findings indicate that monensin has the potential to reduce some mineral requirements of cattle consuming either a grain-based or a forage-based diet. Kirk et al. (1985a) reported that monensin can alter Na and K metabolism in lambs fed a high-grain diet. They observed that apparent absorption of Na and K increased when lambs were fed monensin. They also reported that monensin decreased ileal Na concentrations by 13.8% but did not affect K tissue retention. Kirk et al. (1985b) fed monensin to lambs consuming a high-grain diet and reported that monensin could increase apparent retention of Mg by 52.4%, and apparent retention of Zn by 45%. Kirk et al. (1985b) proposed that the increase in Mg could be due to increased sodium-potassium ATPase-dependent Mg absorption mechanism, once monensin can drive sodium-potassium pumps. The increase in Mg absorption and retention driven by monensin could help prevent hypomagnesemia due to the low content and availability of Mg in forages (Kirk et al., 1985b).

The influence of direct-fed microbials on mineral metabolism is not yet clearly understood. Gordon et al. (2013) reported no improvements in digestibility of Cu, Zn, Mn, Co, and Se when horses were fed yeast cultures and direct-fed microbials. Abas et al.

(2007) observed that supplementation of direct-fed microbial to lambs could cause an increase in serum Zn levels. Swyers (2007) reported increased Cu and Fe digestibility when horses were fed lactic acid bacteria-based direct-fed microbial. Latorre et al. (2015) observed improvements in bone breaking strength and bone composition when supplementing direct-fed microbial to broiler chicken. These studies demonstrate the potential for improvements in mineral absorption, utilization, and digestibility with direct-fed microbial supplementation.

2.9. Conclusion

There is a growing concern and limitation on the use of antibiotic feed additives. It is crucial to find alternative supplements to reduce and replace the use of antibiotics while maintaining animal and economic advancements. Programmed Nutrition Beef Finisher utilizes microbial fermentation extracts as a possible alternative supplement for antibiotics. It appears that factors such as type of diet, grain processing method, intake level, duration of supplementation, and level of supplementation are some of the factors influencing the response to microbial supplements. More studies are required to better understand the range of responses obtained when utilizing microbial products *in vivo* and *in vitro*.

CHAPTER 3.

Mechanisms of Programmed Nutrition in finishing cattle *in vivo*

3.1. Introduction

Programmed Nutrition Beef Program (Alltech Biotechnology Inc., Nicholasville, KY) is a nutritional program consisting of Programmed Nutrition Beef Receiver and Programmed Nutrition Beef Finisher, which are formulated to contain proteinated trace minerals, ascorbic acid, selenium-yeast, *Lactobacillus acidophilus* and *Enterococcus faecium* fermentation products, and either *Aspergillus niger* (Beef Receiver) or *Aspergillus oryzae* (Beef Finisher) fermentation extracts.

Programmed Nutrition Beef Program can potentially reduce the use of feed additives in conventional feedlot diets such as monensin, an ionophore antibiotic that alters fermentation and controls coccidiosis (Stock et al., 1995) and Tylosin which decreases the incidence of liver abscess (Potter et al., 1985). Rumensin® (monensin) and Tylan® (Tylosin) when fed in combination can reduce dry matter intake (DMI) by 5% and improve feed conversions by 7% (Elanco Study, 2012). Programmed Nutrition consists of supplementing Programmed Nutrition Beef Receiver during the step-up period (14 g/head/day) and Programmed Nutrition Beef Finisher (20 g/head/day) during the remainder of the finishing period. Our study focused on studying the effects of Programmed Nutrition Beef Finisher once this product is used for a longer period of time during the nutritional

program concept and would be more beneficial to understand its mechanism of action. It was observed that Programmed Nutrition could increase body and carcass weight without compromising carcass quality and increase DMI while maintaining feed:gain compared to conventional feed additive programs. However, liver abscess incidence increased in steers consuming Programmed Nutrition (Jennings, 2013; Phelps et al., 2014; Phelps et al., 2015; Swingle and Holder, 2015). The mechanism of these effects is unknown and needs to be better understood.

Phelps et al. (2015) observed that Programmed Nutrition with or without exogenous growth promotants (ExGP) generates similar feedlot performance, carcass characteristics, and fatty acid profiles when compared to conventional feedlot systems (Khan et al., 2009), indicating that it could be an organic alternative to antibiotics. Steers consuming Programmed Nutrition had greater DMI (Programmed Nutrition /EGP-: 9.85 kg/d; Programmed Nutrition /EGP+: 11.09 kg/d) compared to Control (Control/EGP-: 9.86kg/d; Control/EGP+: 10.69 kg/d), but average daily gain (ADG) was not affected (Khan et al., 2009). However, steers fed Programmed Nutrition had a greater incidence of total liver abscess than Control (22.5% vs. 12.6%). Jennings et al. (2014) observed that Programmed Nutrition heifers consumed 0.23 kg/d (8.95 vs. 8.73 kg/d) more than Control animals, and had a 3% lower feed conversion, but presented similar final body weight (Control: 504 kg; Programmed Nutrition: 504 kg) and average daily gain (Control: 1.3 kg/d; Programmed Nutrition: 1.29 kg/d). Liver abscess incidence was similar between the Control (12.5%) and Programmed Nutrition (18.6%), with a higher incidence score of “A” in the Programmed Nutrition (2.5% vs 0.7%).

In a conventional feedlot setting, Swingle and Holder (2015) compared Programmed Nutrition with a contemporary diet using 1928 steers consuming steam-flaked corn and wet distillers grain-based diet. Programmed Nutrition (PN) steers had higher average carcass weights (PN: 417 kg vs Contemporary: 410 kg), dressed yield (PN: 64.91% vs Contemporary: 64.45%), *longissimus* muscle area (PN: 96.3 cm^2 vs Contemporary: 92.5 cm^2), carcass-adjusted final BW (PN: 644 kg vs Contemporary: 634 kg), carcass adjusted ADG (PN: 1.75 kg/d vs Contemporary: 1.70 kg/d) and liver abscess prevalence (PN: 26.5% vs Contemporary: 13.6%).

Supplementing α-amylase to finishing beef cattle can increase weight gain and improve carcass characteristics. Potentially by shifting the production of volatile fatty acids (VFA), increasing butyrate production at the expense of propionate. This shift may occur due to a change in the ruminal microbial population, increasing butyrate-producing bacteria and reducing starch-utilizing bacteria. Elolimy et al. (2018) supplemented 24 crossbreed (Simental x Angus) steers with α-amylase (Amaize; Alltech Inc., Nicholasville, KY) at 5 grams/head/d for 140 days in a finishing diet. Alpha-amylase supplementation modified the gene expression of 21 genes in skeletal muscle, where 14 were up-regulated (NR4A3, PPARGC1A, ATF3, ABRA, FOXO1, ELL2, DKK2, FNIP2, SLC25A33, LLRN1, ZNF385B, SYT4, CYTH1, and MPZ) and 7 down-regulated (MIR186, MIR1-1, LOC100299180, LOC788067, IRF2BPL, LOC785630, and LOC784741) compared to the control group. Similarly, Graugnard et al. (2015) fed 135 cross-bred yearling steers consuming a steam-flaked corn diet supplemented with α-amylase (Amaize) at 5 grams/head/d for 160 days. Adipose tissue was collected from 15 steers at days 70 and 160, and RNA was isolated for analysis. Amaize affected gene expression in the adipose tissue,

down-regulating the gene network associated with beta-hydroxybutyrate metabolism. On day 70, Amaize upregulated growth hormone receptor (GHR) by 1.43-fold, and peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1α) by 1.42-fold, which regulates genes involved in energy metabolism. This indicates that Amaize affects gene expression related to metabolism and cellular development in the adipose tissue, which could improve animal performance or affect carcass quality.

These results suggest that the Programmed Nutrition beef program produces growth responses similar to a conventional feedlot program using Rumensin + Tylan. Also, results suggest it produces greater carcass weight with no differences in feed efficiency. While nutritional management that influences carcass growth without affecting animal performance presents a challenging paradigm to study, we hypothesize that Programmed Nutrition may influence rumen fermentation since performance is equivalent to monensin, a known fermentation modifier. Programmed Nutrition may increase butyrate production at the expense of propionate in the rumen, and differences in carcass weight could be caused by differences in carcass repartitioning. Monensin feeding produces pronounced changes in ruminal fermentation that are responsible for the increase in feed efficiency associated with ionophore feeding.

The objective of this study was to determine the impact of Programmed Nutrition Beef Finisher versus traditional feedlot additives (Monensin + Tylosin) on ruminal metabolic changes impacting diet utilization in steers fed a high concentrate diet. The study focused on rumen metabolism, whole-body nutrient balance, and diet utilization.

3.2. Materials and Methods

3.2.1. Animals and Experimental Design

Eight rumen-cannulated Holstein steers (BW = 363 ± 22 kg) were used in a replicated 4 x 4 Latin square design experiment. The treatments were arranged in a 2 x 2 factorial structure with or without Programmed Nutrition Beef Finisher or Monensin/Tylosin. Animals received control (conventional trace mineral supplement), Programmed Nutrition (PN; Programmed Nutrition Beef Finisher), control + Monensin + Tylosin (MT; Elanco Animal Health, Greenfield, IN), or Programmed Nutrition + Monensin + Tylosin (PNMT) (Table 3.1). Treatment supplements were added to the diet at 75 g/head/day. Each experimental period was composed of 27 days (Table 3.2). The steers were fed a conventional feedlot diet consisting of high moisture corn (60.8%), corn silage (15%), corn gluten feed (11%), dry distiller grains (8.6%) and a supplement (4.6%) (Table 3.3). Diet offered started at 2.0 x NEm and intake was equalized during the adaptation period based on the intake of the lowest consuming treatment. To avoid metabolic disorders such as liver abscesses (Nagaraja et al., 2005) and rumen acidosis (Penner et al., 2007; Steele et al., 2009a) steers were adapted to this diet gradually over 21 days on a common control diet receiving control mineral supplement. All steers received a Revalor S implant (Intervet, Summit, NJ) containing 200 mg of trenbolone acetate and 40 mg estradiol at the beginning of the experiment to stimulate growth and increase nitrogen retention.

3.2.2. Intake and Body Weight

During adaptation (days 1-14) and the sample collection period (days 15-27) steers were fed once daily (0700). Fourteen day treatment adaptation was performed to allow rumen microbiome adjustment. Feed for each experimental period was mixed in a single batch, and stored in barrels in the cooler (4ºC). Dry matter intake (DMI) was determined daily by weighing the feed offered and the orts remaining for each animal. Feedstuffs and orts were sampled daily and stored at 0°C. During the adaptation period intakes were equalized based on the intake of the lowest consuming treatment. Bodyweight (BW) was determined at the start of the experiment and the end of each experimental period.

3.2.3. Ruminal Metabolism

On days 15 and 16 ruminal pH was continuously measured (every 1 min) using the LRCpH data logger system (model LRCpH T5; Dascor, Escondido, CA). Loggers were calibrated using pH buffers 4 and 7 at the beginning and end of each measurement period as described by (Penner et al., 2006). Rumen fluid (50 mL) was collected for 48h at 2h intervals and analyzed for volatile fatty acid (VFA) concentrations, starting at 0800 on day 15 and 0700 on day 16. For VFA concentrations, 5 mL samples of ruminal fluid were placed into centrifuge tubes and 0.5 mL 85 mM 2-ethylbutyrate internal standard was added followed by 0.5 mL 50% metaphosphoric acid. The tubes were then capped, mixed, and frozen overnight (-4ºC). The following day the samples were thawed and centrifuged at 39,000 × g for 20 min before transferring to injection vials. The VFA concentrations were determined using gas chromatography with a flame ionization detector (Agilent

HP6890 Plus GC with Agilent 7683 Series Injector and Auto Sampler; Agilent Technologies, Santa Clara, CA) using a Supelco 25326 Nukol Fused Silica Capillary Column (15 m × 0.53 mm × 0.5 μm film thickness; Sigma/Supelco, Bellefonte, PA). To accomplish the analysis, 0.2 μL of the sample was injected at 110ºC with a 2:1 split. After a 1 min hold, the temperature was increased at 5 ºC /min to 125 ºC for 2 min, and the inlet and injector were set at 260 ºC.

3.2.4. Digestibility, Nitrogen, and Energy Balance

The steers hair was clipped to facilitate the attachment of the urine collection harness and to minimize hair contamination in the fecal samples. On day 17 of each period, animals were moved to individual metabolism stalls and adapted for 1 day. Steers were fitted with urine collection harnesses on day 18 and adapted to total urine and feces collection during day 19. Complete urine and fecal collections were performed for 7 days beginning on day 20 at 0700. Urine was collected by continuous suction using a rubber funnel system attached to the ventral portion of the abdomen to allow the collection of urine into a plastic collection vessel. Urine acidity was reduced to pH < 3 by adding 1 L of a 23.5% solution of H_3PO_4 to the collection vessel to prevent ammonia N loss. Urine output weight was recorded daily. Five percent of the urine was subsampled to contribute approximately 250 g to a composite for each period and steer, which was stored at 0°C until analysis. Fecal collection was accomplished by scraping fecal excretions into a trough behind each animal several times daily. The wet weight of fecal output was recorded at 0700 daily for each steer. Each day's collection was mixed thoroughly, and a subsample

was taken at ten percent to give approximately 500 g wet matter per day. At the end of each sample collection period, feces attached to the animal and the harness were scrapped and collected. After steers are removed from the metabolic stalls, feces attached to the stalls were also scrapped and collected to ensure total fecal collection. Feces scrapped were included in the total fecal output of the last day of sampling. Daily fecal samples were frozen at 0°C until the end of the period then composited by period and steer using a Hobart mixer (Model H-600, Hobart Manufacturing Co, Troy, OH) and stored at 0°C until analysis. On days 20 to 22 animals were placed into indirect calorimetry headbox units to evaluate heat production over 48h as described by Koontz et al. (2010). Inspired and expired air were analyzed for O_2, CO_2, and CH_4 concentrations at 9 min intervals, and whole-body heat production was calculated (Brouwer, 1965). Energy retained in protein was calculated using the N-balance and heat production data, by multiplying retained crude protein (N x 6.25; g/d) by heat combustion of protein (23.8 kJ/g). Energy retained in fat was determined by subtracting total retained energy (RE) and energy retained as protein (McLeod and Baldwin VI, 2000).

Feed and fecal samples were dried at 55°C in a forced-air oven for 48 hours, ground through a 1 mm screen in a Wiley Mill (Model 4; Thomas Scientific, Swedesboro, NJ), and dry matter digestibility was calculated. Dry ground feed and fecal samples were analyzed for dry matter and ash content (procedure 930.15; AOAC, 2005), and for fiber to determine acid detergent fiber (ADF) and neutral detergent fiber (NDF) using the detergent analysis system (Goering and Van Soest, 1970a) modified for the ANKOM 200 fiber unit (ANKOM Technology Corporation, Fairport, NY). Feed and fecal samples were ground frozen along with dry ice through a 1 mm screen in a Wiley Mill (Model 4; Thomas

Scientific, Swedesboro, NJ) to reduce loss of N as ammonia, loss of VFA, and lactic acid. Urine, freeze ground feed and feces were analyzed for carbon and N content using the N:C method on an Elementar varioMAX (Elementar Analysen system, Hanau, Germany) (procedure 990.03; (AOAC, 2005), and for the heat of combustion using bomb calorimetry (Parr 1281 Bomb Calorimeter, Moline, IL). Before combustion, urine samples were oven-dried for 2 days at 55°C soaked on polyethylene bags (Jeb Plastics Inc., Wilmington, DE) where the heat of combustion per gram of polyethylene bag was subtracted from the total heat of combustion to determine urine gross energy (Wilkerson et al., 1997).

Calculations N-Balance:

- Absorbed N (g/d) = Intake N – Fecal N
- Excreted N (g/d) = Fecal N + Urine N
- Retained N (g/d) = Intake N – Excreted N
- Digestible N (%) = [(Intake N (g/d) – N Feces (g/d)) / Intake N (g/d)] * 100

Calculations Energy Balance:

- O_2 consumption (L/kg $BW^{0.75}$/d) = O_2 consumed (L/d) / $BW^{0.75}$
- CO_2 production (L/kg $BW^{0.75}$/d) = CO_2 produced (L/d) / $BW^{0.75}$
- CH_4 production (L/kg $BW^{0.75}$/d) = CH_4 produced (L/d) / $BW^{0.75}$
- Heat production (kcal/d) = 3.869 (LO_2) + 1.195 (LCO_2) - 0.516 (LCH_4) - 1.431 (Urine N; g/d)
- Heat production (kJ/ kg $BW^{0.75}$/d) = Heat production (kJ/d) / $BW^{0.75}$
- Respiration Quotient (RQ) = CO_2 produced (L/d) / O_2 consumed (L/d)
- Intake Energy (kJ/$BW^{0.75}$/d) = [Intake E (g/d) * Feed E (kJ/g)] / $BW^{0.75}$
- Fecal Energy (kJ/$BW^{0.75}$/d) = [Fecal Output (g/d) * Fecal E (kJ/d)] / $BW^{0.75}$

- Digestible Energy (kJ/$BW^{0.75}$/d) = [Intake E - Fecal E] / $BW^{0.75}$
- Digestibility Energy (%) = [(Intake E - Fecal E) / Intake E] * 100
- Gas Energy (kJ/$BW^{0.75}$/d) = [(CH_4 (L/d) * 9.45) * 4.184] / $BW^{0.75}$
- Urine Energy (kJ/$BW^{0.75}$/d) = [Urine Output (g/d) * Urine E (kJ/d)] / $BW^{0.75}$
- Metabolizable Energy (kJ/$BW^{0.75}$/d) = [Digestible E - (Urine E + Gas E)] / $BW^{0.75}$
- Retained E (kJ/$BW^{0.75}$/d) = [Metabolizable E (kJ/d) - Heat production (kJ/d)] / $BW^{0.75}$
- Energy retained in protein (kJ/$BW^{0.75}$/d) = [(N Retained (g/d) * 6.25) * 23.8] / $BW^{0.75}$
- Energy retained in fat (kJ/$BW^{0.75}$/d) = [Retained E - Energy retained in protein] / $BW^{0.75}$

3.2.5. Protein Turnover

Protein turnover was estimated using the ^{15}N-glycine end-product method described previously (Wessels et al., 1997). On day 18, the animals were fitted with the urine collection harnesses, at 1500 an aliquot of urine was saved for background ^{15}N analysis. On day 19 at 0700 (same time as feeding) each steer received a single dose (5 mg/kg BW) of ^{15}N-glycine (Andover, MA, Cambridge Isotope Laboratories, Inc.) in 100 mL of water administered abomasally, and flushed with an additional 200mL of water after dosing to ensure complete infusion. From day 20 at 0700 through day 23 at 0700 an additional 100 grams (maximum) of acidified urine from each steer was sampled and frozen for later analysis of ^{15}N urea at each 24 h collection.

Urea nitrogen content of urine samples obtained for ^{15}N analysis was analysed using the Technicon Auto-Analyzer (Bran + Luebbe, Buffalo Grove, IL, USA) as described previously by Marsh et al. (1965). After determination of urea content, individual urine

samples collected from day 20 through day 23 were processed and analysed for ^{15}N enrichment as described previously by Reeder et al. (2011) with modifications, to ensure 80% or more urea recovery from the column. Briefly, after determination of the urea content of the samples, the urine was centrifuged for 10 minutes at 1,000 x g at 4˚C. An aliquot of urine containing approximately 100 μmol of urea was mixed with 4.0 mL of water, the pH was adjusted to 2.5, and it was poured onto a 1.8 mL AG 50W-X8, 100-200 mesh, and H^+ form (Bio-Rad Laboratories, Richmond, CA, USA) cation exchange column. The first 10 mL of filtrate was discarded and then 30 mL of water was added to the column and the eluent collected. The samples were then dried for 3 days in a 55˚C oven and reconstituted with 2mL 0.1 M pH 7.0 phosphate buffer. Following reconstitution, 100uL of the sample was placed in a 25 mL Erlenmeyer flask with 3 mL of 0.1 M pH 7.0 phosphate buffer. A 5 x 25mm strip of Whatman 934-AH filter paper was placed in a stopper cup (VWR International, Radnor, PA, USA) and 30 μL of 2.5 M $KHSO_4$ was added to the filter paper to trap ammonia. Urease (Sigma urease type III from Jack Beans 680unit/mL; 0.2 mL 100 unit/mL) was then added, and the flask was immediately stoppered. The flasks were incubated for 1 hour at 25˚C in a Dubnoff shaking incubator. Two hundred microliters of 3 N NaOH were injected through the stopper into the flask and the flasks were shaken for an additional hour and then allowed to stand for 24 hours. The stoppers were then removed and placed in a desiccator containing an open container of concentrated H_2SO_4 and allowed to dry for an additional 24 hours. When the filter papers were dry, they were placed in an opened Sn cup and the cup was folded around the filter papers in preparation for analysis. Samples were then sent to the University of California, Davis Stable Isotope Facility and placed in a PDZ Europa Anca Sample Preparation Unit to be

analyzed for ^{15}N using a PDZ Europa 20-20 Isotope Ratio Mass Spectrometer. Protein turnover was calculated using ^{15}N-glycine as a marker as described previously (Waterlow et al., 1978)

Calculations Protein Turnover:

- Urea excreted in urine (g/d) = [(urea urine; mmol/L) * (60.06 g; urea molecular weight) * (urine output; L)] / 1000
- N excreted as urea in urine (g N/d) = [(urea urine; mmol/L) * (28 g; 2 N per urea molecule; 14 g N molecular weight) * (urine output; L)] / 1000
- ^{15}N atom excess (%) = (^{15}N atom excess) – (^{15}N atom excess background)
- ^{15}N urine (g/d) = [(^{15}N atom excess; %) * (urea excreted in urine; g/d)] / 100
- Cumulative ^{15}N urine (g/d) = average ^{15}N urine (g/d) days 20 to 23
- Corrected ^{15}N-glycine dose (g/d) = ^{15}N-glycise dose * (0.18421; 18.421% N in glycine)
- ^{15}N Fractional recovery = (Corrected ^{15}N-glycine dose; g/d) / (Cumulative ^{15}N urine average days 20 to 23; g/d)
- Protein turnover (g N/d) = (N excreted as urea in urine average days 20 to 23; g N/d) / (^{15}N Fractional recovery)
- Protein synthesis (g N/d) = (Protein turnover; g N/d) – (Urine urea N; g N/d)
- Protein degradation (g N/d) = (Protein turnover; g N/d) – (Absorbed N; g N/d)

3.2.6. Blood Plasma Analysis

On day 26 at 1400 animals were fitted with a jugular catheter (14 gauge, 6 inch) for blood sample collection and analysis of plasma insulin. On day 27 blood samples were collected at 0h, 2h, 4h and 6h post-feeding into a heparinized syringe and stored in vacutainers (Becton, Dickson and Co., Franklin Lakes, NJ), immediately placed on ice,

centrifuged at 1,200 × g for 25 min at 4°C and stored at −20°C until further analysis. Plasma insulin was analyzed in duplicate using ^{125}I-labeled insulin radioimmunoassay (RIA) kit (P1-12K; Millipore Corporation, Billerica, MA) (DiCostanzo et al., 1999). The assay was validated via linearity of dilution and recovery of spiked samples for variation within and between assays. Intra-assay and inter-assay coefficient of variation (CV) were respectively 3.67% and 1.30%.

3.2.7. Statistical Analysis

The experimental model used was a replicated Latin square design analyzed with mixed models of SAS (SAS version 9.4; SAS Inst. Inc., Cary, NC), where steer was the experimental unit. Volatile fatty acid, rumen pH and blood plasma insulin data were analyzed using PROC MIXED with repeated measures over time. Mean differences were analyzed using least significant difference (LSD) feature of SAS. Treatments and interactions were considered different when $P < 0.05$ and a trend when $P < 0.10$.

3.3. Results

3.3.1. Intake, Digestibility, and Gain

There was no difference ($P > 0.05$) between treatments in dry matter (DM) intake and digestibility, organic matter (OM) intake and digestibility, dry matter and organic matter fecal output, water intake, urine output, and average daily gain (ADG) (Table 3.4).

Neutral detergent fiber (NDF) intake and fecal output did not differ, but there was a tendency for lower NDF digestibility in animals consuming PN when compared to MT (PN; P = 0.0955). Animals consuming MT had 9.15% higher NDF digestibility than animals consuming PNMT (Table 3.5). There was a tendency for higher acid detergent fiber (ADF) fecal output for PNMT (PN x MT; P = 0.0815). Similar to NDF digestibility, ADF digestibility was lower (PN x MT; P = 0.0342) in animals consuming PNMT, where ADF digestibility was 16.84% higher in MT when compared to PNMT (Table 3.5).

3.3.2. Ruminal Metabolism

Rumen pH was not different among treatments (P > 0.05), varying between 6.21 to 5.71 (Table 3.6) (Figure 3.1). Time below rumen pH 5.6 (MT; P = 0.0081) and 5.3 (MT; P = 0.0242) were significantly higher when MT was present, where PNMT stayed below ruminal pH 5.6 and 5.3 longer than the other treatments. Treatment PNMT stayed below rumen pH 5.6 for 24.81 hours (1488.75 minutes) and below rumen pH 5.3 for 11.31 hours (678.75 minutes) (Table 3.6). Rumen total volatile fatty acid (VFA) concentration and acetate:propionate were not different between treatments (P = 0.7409) (Table 3.7). There was a significant treatment effect for rumen valerate concentration (P = 0.0125) and molar proportion (P = 0.0235) (Table 3.7). Control had the highest valerate concentration (3.95 mM) and valerate molar proportion (3.27 moles/ 100 moles), while MT had the lowest (2.32 mM and 1.99 moles/100 moles) (Table 3.7). There was a significant Treatment x Time interaction for butyrate molar proportion (P = 0.0010) (Table 3.7) (Figure 3.2), with a trend for Treatment x Time interaction for butyrate concentration (P = 0.0601) (Table

3.7) (Figure 3.3). Treatment MT presented the highest butyrate molar proportion at time of feeding (9.24 moles/100 moles). Compared to other treatments, PNMT had the lowest butyrate molar proportion 8 to 18 hours (hour 15 to 1) after feeding (hour 7), with a rapid increase at the next feeding time (hour 7). Meanwhile, Control had the highest butyrate molar proportion 4 to 14 hours after feeding (hour 11 to 21), with a rapid decrease at the next feeding time (hour 7).

3.3.3. Nitrogen Balance

There was no difference in N intake across treatments ($P > 0.05$) (Table 3.8). Nitrogen digestibility obtained was 32.58% for MT, 33.12% for Control, 34.38% for PN, and 39.53% for PNMT ($P > 0.05$) (Table 3.8). Although MT inclusion lowered fecal N output ($P = 0.0092$), there were no differences in N excreted, N absorbed, N retained, or N digestibility between treatments ($P > 0.05$) (Table 3.8).

3.3.4. Indirect Calorimetry and Energy Balance

Oxygen (O_2) consumption and carbon dioxide (CO_2) production had significant PN x MT interactions ($P < 0.001$) (Table 3.9). These parameters were higher when MT or PN were consumed separately but were lower when consumed in combination (PNMT). Consumption of O_2 was 11.29% higher for PN than Control, and production of CO_2 was 12.21% higher for MT than Control (Table 3.9). There were no differences in CH_4 production between the treatments ($P > 0.05$) (Table 3.9). There was also a significant PN

x MT interaction ($P < 0.001$) for heat production, which was highest for PN followed by MT, but lower for PNMT (Table 3.9). Respiration quotient (RQ) was significantly higher when MT was present (P 0.0008) (Table 3.9).

There were no differences between treatments on intake energy, digestible energy, gas energy, metabolizable energy, and energy retained in protein ($P > 0.05$) (Table 3.9). Fecal energy tended to be lower when MT was present ($P = 0.0833$) (Table 3.9). There was also a tendency for PN x MT interaction in urine energy ($P = 0.0811$), where PNMT presented the lowest energy in urine (Table 3.9). Retained energy presented a significant PN x MT interaction ($P = 0.0035$), where PNMT was 17.37% greater than PN and 17.74% greater than MT (Table 3.9). There was also a significant PN x MT interaction ($P = 0.0026$) for energy retained in fat, which was 20.16% higher in PNMT than PN, and 18.39% higher in PNMT than MT (Table 3.9).

3.3.5. Protein Turnover

There were no differences between treatments on ^{15}N fractional recovery and protein synthesis ($P > 0.05$) (Table 3.10). There was a higher urea excretion rate when animals consumed MT ($P = 0.0384$), where the rate was 18.68% higher for MT than Control (Table 3.10). There was a tendency for higher N excreted as urea was observed when animals consumed MT ($P = 0.0674$) (Table 3.10). There was a tendency for a PN x MT interaction for protein turnover ($P = 0.0715$) and protein degradation ($P = 0.0825$) (Table 3.10).

3.3.6. Blood Plasma Insulin

There were no significant Treatment or Treatment x Time differences in blood plasma insulin concentration among treatments ($P > 0.05$) (Table 3.11). Blood plasma insulin obtained was 38.90 uU/mL for PNMT, 38.93 uU/mL for MT, 40.45 uU/mL for PN, and 50.02 uU/mL for Control ($P > 0.05$) (Table 3.11).

3.4. Discussion

Dry matter intake (DMI) was not different among treatments, which was due to the equalization of intake at 2.0 x NEm. The inclusion of Programmed Nutrition Beef Finisher at 75 g/head/day did not generate differences in average daily gain (ADG). This is in agreement with Jennings et al. (2014) and Phelps et al. (2015), where no differences in final body weight were observed when supplementing cattle with Programmed Nutrition Beef Finisher. A meta-analysis on the effects of monensin in beef cattle on feed efficiency concluded that in forage-based diets, monensin might affect average daily gain without reducing dry matter intake. However, in finishing diets with higher energy concentration than forage-based diets, monensin might have less of an effect on improving average daily gain and more of an effect reducing dry matter intake (Duffield et al., 2012). In our study, the lack of increase in average daily gain in monensin supplemented animals is in agreement with the results demonstrated in the meta-analysis (Duffield et al., 2012).

Dry matter and organic matter digestibility did not differ between the treatments, which was also observed by Meyer et al. (2009) when supplementing steers with monensin and tylosin in combination. Muntifering et al. (1980) supplemented monensin to steers fed

a high-concentrate and high-protein diet, and DM digestibility was not different between the monensin supplemented and the control group. This was attributed to the high level of protein in the diet and equalized intakes. Wedegaertner and Johnson (1983) detected an increase in DM digestibility when supplementing steers with monensin, but this increase was mostly driven by a rise in neutral detergent fiber (NDF) digestibility. In this study, we did not obtain an increase in NDF digestibility when animals were fed monensin and tylosin, which could have contributed to the absence of higher DM digestibilities.

In this study, ADF digestibility was higher when monensin and tylosin (MT) were supplemented to the animals, but lowest when monensin and tylosin were supplemented in combination with Programmed Nutrition (PNMT). The effects of microbial supplementation in ruminal and animal performance parameters can vary greatly, depending on the type of diet, level of intake, and even time of the year (Dawson et al., 1990; Oellermann et al., 1990; Newbold et al., 1992a; Beharka and Nagaraja, 1993; Caton et al., 1993). Varel and Kreikemeier (1994a) did not observe differences in NDF digestibility when supplementing *Aspergillus oryzae* fermentation extract fed to cows consuming alfalfa and bromegrass diets. Even though *Aspergillus oryzae* contains a high level of fibrolytic enzymes, which should improve fiber digestion in the rumen, the fibrolytic effect of *Aspergillus oryzae* could differ depending on the structure of the carbohydrates present in the diet (Newbold et al., 1992a). Meanwhile, Caton et al. (1993) reported a decrease in NDF and ADF digestibility when supplementing steers with *Aspergillus oryzae* fermentation extract in June and August, but an increase in NDF and ADF digestibility occurred when supplementing in July. The reasons for differences in

response according to the time of the year are unclear, which emphasizes how these supplements can generate inconsistent responses.

No differences in rumen pH between treatments were observed in this study, but the time below rumen pH 5.6 and 5.3 increased when PNMT was supplemented. Felix et al. (2012) obtained similar results where increasing levels of monensin (0, 22, 33, and 44 mg/kg DM) did not improve ruminal pH over time. It was surprising that this study did not detect higher pH values when monensin was supplemented. It has been demonstrated that this ionophore can maintain higher ruminal pH, where Nagaraja et al. (1981) administered monensin intraruminally twice at 1.3 mg/kg BW, and Burrin and Britton (1986) fed monensin at 0, 150, or 300 mg/head/day. Other studies observed no change in ruminal pH and time below rumen pH 5.6 when supplementing monensin to cattle (28.6 mg/kg DM) (Erickson et al., 2003). The differences in response could be due to different levels of monensin supplemented, as studies that observed higher rumen pH offered monensin at higher rates.

The effect of microbial fermentation extracts on ruminal pH can also vary. Nascimento et al. (2017) did not observe differences in ruminal pH when supplementing *Aspergillus oryzae* to feedlot cattle. Nocek et al. (2002) demonstrated that *Enterococcus faecium* and *Lactobacillus* supplementation to early lactation cows could increase rumen pH. The presence of these lactate-producing bacteria could potentially cause the rumen microorganisms to adapt to the lactic acid in the rumen.

Monensin can improve ruminal fermentation efficiency by reducing energy lost during VFA production (Poos et al., 1979). By shifting the VFA profile to propionate,

monensin can decrease the energy lost as CH_4 during acetate and butyrate synthesis (Hungate, 1966). Richardson et al. (1976) observed that increasing concentrations of monensin did not change total VFA concentrations but decreased valerate concentrations while increasing propionate concentrations both *in vitro* and *in vivo*. No differences in total VFA (mM) were observed between the treatments in this study, but there was a significant decrease in valerate concentration and molar proportion when monensin and tylosin were supplemented. Previous studies also observed a depression in valerate production when supplementing monensin to cattle (Raun et al., 1976; Utley et al., 1977; Horton et al., 1980; Wood et al., 2016; Polizel et al., 2017). Decreases in valerate concentration and molar proportion could be correlated to the metabolism of cellulolytic bacteria, once isoacids like valerate are consumed by these bacteria in the rumen (Bentley et al., 1955; Andries et al., 1987). In this study, monensin did not cause an increase in propionate. Although monensin generally causes a reduction in rumen butyrate proportion (Burrin and Britton, 1986), we observed higher butyrate molar proportions over time when MT or PN was fed, with a decrease in this parameter when fed in combination (PNMT). There is a high variation in the monensin response to ruminal butyrate concentrations (Ellis et al., 2012). Differences in ruminal microbial population and the inhibitory effect of monensin upon these populations could contribute to the varying butyrate responses (Henderson et al., 1981; Dijkstra, 1994; Johnson and Johnson, 1995; Ellis et al., 2012). Kenney et al. (2015a) observed that supplementing steers with a mixed bacterial culture that consisted primarily of *Lactobacillus acidophilus* and *Enterococcus faecium* did not change total VFA concentrations, but significantly increased acetate proportion. Beauchemin et al. (2003) also did not observe differences in total VFA concentrations when supplementing steers

with *Enterococcus faecium*. They did not observe an increase in acetate concentration, but detected an increase in propionate concentration and a decrease in butyrate concentration. These effects were attributed to the capacity of lactate-producing bacteria to stimulate the growth of lactic acid-utilizing bacteria, which produce propionate.

Monensin can inhibit the growth of lactate-producing bacteria (Poos et al., 1979), which are components of Programmed Nutrition. Studies using *Aspergillus oryzae* fermentation extract generally do not detect differences in total VFA concentration and VFA profile (Oellermann et al., 1990; Newbold et al., 1991; Varel and Kreikemeier, 1994a; Varel and Kreikemeier, 1994b).

Nitrogen balance was generally not affected by treatment in this study. Only fecal N output was reduced when monensin and tylosin were present. Monensin can reduce ruminal protein degradation *in vitro* (Van Nevel and Demeyer, 1996). This effect is attributed to the inhibitory effect of monensin on amino acid-fermenting bacteria (Krause and Russell, 1996). Monensin can also decrease microbial protein flow to the small intestine due to its inhibitory effect on rumen protein degradation (Bergen and Bates, 1984). Beede et al. (1986) obtained higher apparent N digestibility when supplementing growing steers fed a low-protein diet (8.72% CP, DM-basis). Muntifering et al. (1980) did not observe differences in N balance when supplementing monensin to steers consuming a sorghum-based diet (11.7% CP, DM-basis). These diets were not designed to be protein deficient (15.4% CP, DM-basis), which could have led to the lack of improvement in N digestibility by monensin. Monensin can improve N retention and digestibility by reducing urinary and fecal N excretion and increasing protein absorption from the small intestine (Dinius et al., 1976; Joyner Jr. et al., 1979; Russell and Strobel, 1989).

Effects of monensin on heat production (HP) can vary; some studies reported an increase in HP (Wedegaertner and Johnson, 1983), while others did not detect any differences (Thornton and Owens, 1981; Rumpler et al., 1986; Boardman et al., 2016; Hemphill et al., 2018) when supplementing monensin to cattle. Joyner Jr. et al. (1979) observed higher digestible energy and HP when wethers were supplemented with 20 ppm of monensin but not when supplemented with 10 ppm. Our study observed an increase in heat production when monensin and tylosin (MT) or Programmed Nutrition (PN) was supplemented, with a decrease in this parameter when PNTM was supplemented.

It has been hypothesized that lactate-producing bacteria would stimulate the growth of lactate-utilizing bacteria, generating an increase in propionate production and consequently reducing CH_4 production (Piveteau, 1999). However, Jeyanathan et al. (2016) demonstrated that *Lactobacillus bulgaricus* can decrease CH_4 production without changing propionate concentrations *in vitro*, but this effect was not observed for *Lactobacillus pentosus*. The decrease in CH_4 production without affecting propionate concentrations observed by Jeyanathan et al. (2016) suggests that other factors could influence the CH_4 response. Various responses were reported with different *Lactobacillus* species, indicating that distinct species can have several effects on CH_4 and VFA dynamics in the rumen. Although monensin can reduce CH_4 production (Wedegaertner and Johnson, 1983; Hemphill et al., 2018), the microbial population can adapt to monensin after 9 days of feeding, where there is no longer a reduction in CH_4 concentrations (Dinius et al., 1976). Our study used a replicated Latin Square design with 14-day diet adaptation period, which creates a possibility of ruminal adaptation to monensin over time.

Respiration quotients (RQ) greater than 1.0 indicate an accumulation of fat by the animal (Blaxter and Wainman, 1966; Ebert et al., 2017). This study observed that monensin and tylosin (MT) increased RQ when fed with or without Programmed Nutrition (PN). This differs from Thornton and Owens (1981), where no effect of monensin on RQ was observed. Our data did not support a shift towards more fat-deposition by the animal with supplementation of monensin, as we did not detect differences in energy retained as protein, and detected lower fat deposition when animals were supplemented with monensin and tylosin compared to control. This reduction in energy retained in fat compared to control was lower when monensin and tylosin were supplemented in combination with Programmed Nutrition. The differences in heat production could be a result of numerically differences in urine energy among treatments. Although monensin and tylosin produced a tendency for lower fecal energy loss, the treatments supplemented in this study did not produce differences in gaseous energy, digestible energy (DE), or metabolizable energy (ME). Control presented the highest retained energy (RE) and energy retained in fat when compared to the other treatments. Retained energy and energy retained in fat parameters decreased when animals were supplemented with monensin and tylosin or Programmed Nutrition, but this reduction was less acute when they were fed in combination (PNMT). These results is not in agreement with Joyner Jr. et al. (1979) where monensin increased DE, RE, and ME in lambs, but is in agreement with Boardman (2015) and Hemphill et al. (2018) where no differences in gaseous energy, DE, and ME were detected when supplementing cattle with monensin.

Protein turnover estimates are based on the metabolism of all amino acids in the body when using ^{15}N-labeled tracers (Waterlow et al., 1978). When Programmed Nutrition

Beef Finisher was fed in combination with monensin and tylosin, there was a tendency for lower whole-body protein turnover and protein degradation rates. An unbalanced supply of amino acids can limit protein synthesis (Everson et al., 1989), but this effect is rarely a concern in cattle-fed high-grain diets (Wright and Loerch, 1988; Hussein and Berger, 1995). Increases in protein supply above the requirement have a minimal impact on protein turnover (Salter et al., 1990). Our study offered a diet to animals containing 60% high-moisture corn and was not designed to be limiting in amino acid content, so these factors should not influence the differences in protein estimates observed. The higher levels of urea excretion in urine when monensin and tylosin were supplemented did not cause changes in ^{15}N fractional recovery, protein turnover, protein synthesis, or protein degradation. Feeding N above requirements can increase urea excretion (Reynolds and Kristensen, 2008).

The effect of direct-fed microbials on whole-body protein turnover is not yet clearly understood. In this study, feeding microbial fermentation extracts increased protein turnover rate numerically but not statistically. However, feeding these microbials in combination with monensin and tylosin caused a decrease in these parameters, which could result from the inhibitory effect monensin has on specific microbial populations. Monensin has the potential to inhibit lactate-producing bacteria, such as *Lactobacillus acidophilus* and *Enterococcus faecium*, both of which are components of Programmed Nutrition. Other studies have hypothesized the inhibitory effect of monensin on the ruminal microbial population. Poos et al. (1979) reported a reduction in bacterial nitrogen flow to the abomasum when monensin was fed. Hanson and Klopfenstein (1979) hypothesized that the observed protein-sparing action with monensin supplementation could be due to a

reduction in ruminal microbial activity. In our study, plasma insulin levels were not altered by the addition of Programmed Nutrition or monensin and tylosin. Similar to our findings, Vendramini et al. (2015) did not detect differences in plasma insulin levels when supplementing heifers with monensin. However, Raun et al. (1976) observed increased plasma insulin when cattle were fed monensin at 22 ppm but not when fed 2.7, 5.5, 11, 33, 44, or 88 ppm. The increase in plasma insulin could be expected if propionate, butyrate, or valerate production in the rumen were increased (Bergen and Bates, 1984). In this study, addition of MT or PN did not change propionate in the rumen, and caused a decrease in ruminal valerate concentration. Higher energy intake is associated with higher circulating insulin levels (Cappellozza et al., 2014). As previously mentioned, dry matter intake was equalized across treatments in this study, which eliminates the possibility of higher energy intake among treatments. These factors could explain the lack of differences in insulin concentration across the treatments.

3.5. Conclusions

Supplementing Programmed Nutrition Beef Finisher, monensin and tylosin, or their combination, did not improve dry matter digestibility, average daily gain, ruminal total VFA production, nitrogen digestibility, methane production, and blood plasma insulin levels. Programmed Nutrition Beef Finisher has the potential to decrease NDF digestibility when supplemented to steers consuming a high-grain diet. Supplementing Programmed Nutrition Beef Finisher in combination with monensin and tylosin can cause a reduction in ADF digestibility. Still, this parameter can be improved when only Programmed Nutrition

Beef Finisher is supplemented. Programmed Nutrition Beef Finisher does not enhance overall rumen pH. Feeding this supplement in combination with monensin and tylosin can increase the time rumen pH is below 5.6 and 5.3, which could lead to adverse effects of ruminal acidosis. When fed in combination with monensin and tylosin, Programmed Nutrition Beef Finisher can decrease the rate of protein turnover, protein synthesis, and protein degradation.

Table 3.1. Composition of treatments Control (C; conventional trace mineral supplement), Program Nutrition (PN; Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY), Monensin + Tylosin (MT; Elanco Animal Health, Greenfield, IN), and Program Nutrition + Monensin + Tylosin (PNMT) offered to animals on an as-fed basis (75 g/head/day).

Item	Control[1]		Programmed Nutrition[2]	
	C	MT	PN	PNMT
Moisture, %	1.4	1.2	1.4	1.4
Dry Matter, %	98.6	98.8	98.6	98.6
Crude Protein, %	0.8	1.1	7.0	7.6
Calcium, %	26.63	26.75	27.67	27.10
Phosphorous	0.01	0.02	0.15	0.16
Magnesium, %	0.31	0.32	0.35	0.33
Potassium, %	0.09	0.08	0.30	0.31
Sodium, %	0.45	0.08	0.60	0.105
Sulfur, %	1.40	1.43	0.57	0.57
Chloride Ion, %	3.90	0.06	0.08	0.09
Iron, ppm	525	631	565	784
Zinc, ppm	10,900	11,200	1,200	1,200
Copper, ppm	2,210	2,340	309	309
Manganese, ppm	5,300	5,410	876	841
Molybdenum, ppm	< 0.1	0.3	1.0	0.9
Cobalt, ppm	58.45	32.98	144.18	150.11
Iodine, ppm	64	64	66	66
Selenium, ppm	38.7	38.0	37.0	37.0
Vitamin A, IU/kg	283,640	283,640	283,640	283,640
Vitamin D_3, IU/kg	35,450	35,450	35,450	35,450
Monensin, g/kg	0	6.0	0	6.0
Tylosin, g/kg	0	1.2	0	1.2

C: Control; conventional trace mineral supplement
MT: Control + Monensin + Tylosin
PN: Programmed Nutrition Beef Finisher
PNMT: Programmed Nutrition Beef Finisher + Monensin + Tylosin
[1]Control ingredients: Calcium carbonate, rice hulls, zinc sulfate, manganese sulfate, copper sulfate, mineral oil, grain products, sodium selenite, vitamin A supplement, vitamin D_3 supplement, calcium iodate, cobalt sulfate.
[2]Programmed Nutrition ingredients: Calcium carbonate, hydrolyzed yeast, plant protein products, selenium yeast, zinc proteinate, manganese proteinate, cobalt proteinate, processed grain by-products, hydrated sodium calcium aluminosilicate, copper proteinate, ascorbic acid (vitamin C), dried *Aspergillus oryzae* fermentation extract, calcium iodate, dried *Lactobacillus acidophilus* fermentation product, dried *Enterococcus faecium* fermentation product, rice hulls, mineral oil, grain products, vitamin A supplement, vitamin D_3 supplement.

Table 3.2. Timeline for each experimental period (4 periods total) of in vivo study.

Day	Hour	Activities
1 to 14	0700	Diet adaptation period
15	0800 - 2h intervals	Ruminal pH measured continuously Rumen Fluid (VFA)
16	0700 - 2h intervals	Ruminal pH measured continuously Rumen Fluid (VFA)
17	0700	Body weight recorded for ^{15}N dosage Metabolic stall adaptation
18	0700 1500	Urine collection harness fitted Aliquot of urine (background ^{15}N analysis)
19	0700	Adaptation total urine and feces collection Abomasal single dose of ^{15}N-glycine
20	0700	Total urine and feces collection Day 1 100g of urine collected (^{15}N urea analysis) Animals placed into indirect calorimetry headbox
21	0700	Total urine and feces collection-Day 2 100g of urine collected (^{15}N urea analysis) Animals in indirect calorimetry headbox
22	0700	Total urine and feces collection-Day 3 100g of urine collected (^{15}N urea analysis) Animals removed from indirect calorimetry headbox
23	0700	Total urine and feces collection-Day 4 100g of urine collected (^{15}N urea analysis)
24	0700	Total urine and feces collection-Day 5
25	0700	Total urine and feces collection-Day 6
26	0700 1400	Total urine and feces collection-Day 7 Animals fitted with a jugular catheter
27	0 hours postfeeding	Body weight recording
	0, 2, 4, 6 hours postfeeding	Blood sample collections

Table 3.3. Composition of diet offered to animals at 2.0 x NEm in in vivo study.

Ingredient	% of As Fed	% of DM
High Moisture Corn	57.17	60.80
Corn Silage	25.01	15.00
Corn Gluten Feed	8.28	11.00
Corn DDGS	6.40	8.60
Limestone	0.68	1.00
Urea	0.68	1.00
Tallow	0.68	1.00
Treatment Supplement	0.60	0.87
Salt	0.28	0.41
Potassium Chloride	0.17	0.25
Magnesium Oxide	0.05	0.07
Nutrient Concentration	**As Fed**	**DM**
Moisture, %	37.2	-
Dry Matter, %	62.8	-
Crude Protein, %	9.6	15.4
Soluble Protein, % CP	-	55
ADF, %	5.1	8.1
NDF, %	10.0	15.9
Lignin, %	1.4	2.2
NFC, %	37.0	58.9
Crude Fat, %	3.5	5.5
Ash, %	2.72	4.33
TDN, %	52.0	83.0
NEm, Mcal/kg	1.32	2.09
NEg, Mcal/kg	0.90	1.43
Calcium, %	0.21	0.34
Phosphorous, %	0.30	0.48
Magnesium, %	0.11	0.18
Potassium, %	0.54	0.86
Sodium, %	0.126	0.200
Sulfur, %	0.11	0.18
Iron, ppm	85	136
Zinc, ppm	23	37
Copper, ppm	3	5
Manganese, ppm	8	13
Molybdenum, ppm	1.4	2.3

Table 3.4. Least square means of feed intake and digestibility parameters, water intake and average daily gain in steers supplemented with Program Nutrition Finisher (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	MT	PN	PN x MT
Intake As-fed, kg/d	13.35	13.27	13.07	13.15	0.55	0.1921	0.9837	0.6107
DMI, kg/d	8.84	8.79	8.65	8.70	0.37	0.2966	0.9701	0.6942
DM Fecal Output, kg/d	2.21	2.11	2.05	2.08	0.13	0.1748	0.6178	0.3542
DM Digestibility, %	74.69	75.73	76.35	75.75	1.83	0.1521	0.6952	0.1622
OM Intake, kg/d	12.73	12.65	12.46	12.53	0.53	0.1918	0.9862	0.6120
OM Fecal Output, kg/d	7.16	7.04	6.98	6.75	0.44	0.4283	0.5520	0.8623
OM Digestibility, %	43.48	44.08	43.89	45.88	4.09	0.6186	0.5602	0.7542
Water Intake, L/d	28.12	28.65	28.92	27.08	2.19	0.8338	0.7180	0.5170
Urine Output, kg/d	8.89	9.76	8.71	9.47	0.92	0.7019	0.2825	0.9804
ADG, kg/d	1.63	1.42	1.58	1.74	0.16	0.3399	0.8509	0.2161

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

Table 3.5. Least square means for neutral detergent fiber (NDF) and acid detergent fiber (ADF) parameters in steers supplemented with Program Nutrition Finisher (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	MT	PN	PN x MT
NDF Intake, g/d	2025.58	2010.23	2005.18	1992.72	97.01	0.7976	0.8508	0.9844
NDF Fecal Output, g/d	824.59	879.95	802.73	909.03	55.41	0.9415	0.1126	0.6055
NDF Digestibility, %	58.81	55.83	59.54	54.09	2.92	0.8342	0.0955	0.6129
ADF Intake, g/d	830.33	824.52	821.85	817.28	43.16	0.8217	0.8817	0.9859
ADF Fecal Output, g/d	419.80	386.62	376.07	450.08	32.50	0.7380	0.4910	0.0815
ADF Digestibility, %	48.87[ab]	52.61[ab]	54.03[a]	44.93[b]	3.61	0.6588	0.3521	0.0342

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

[ab]Least squares means that do not contain at least one of the same letters across rows and columns differ ($P < 0.05$).

Table 3.6. Least square means for rumen pH parameters in steers supplemented with Program Nutrition Finisher (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	Treatment	Time	Treatment x Time
Rumen pH	6.21	6.06	5.85	5.71	0.14	0.1810	< 0.001	0.7170
						MT	PN	PN x MT
Rumen pH < 5.6, min	423.75[b]	622.50[b]	1260.00[ab]	1488.75[a]	295.89	0.0081	0.4708	0.9594
Rumen pH < 5.3, min	127.50[b]	322.50[ab]	506.25[ab]	678.75[a]	160.26	0.0242	0.2381	0.9414

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

[ab]Least squares means that do not contain at least one of the same letters across rows and columns differ (P < 0.05).

Table 3.7. Least square means for rumen volatile fatty acids (VFA) in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Control	PN	M T	PNMT		P-value		
Item	millimoles/L				SEM[1]	Treatment	Time	Treatment x Time
Acetate	55.69	59.49	57.60	55.00	4.70	0.8569	< 0.001	0.8484
Propionate	44.41	48.86	42.62	45.24	5.39	0.5750	< 0.001	0.9917
Butyrate	8.95	10.25	9.29	8.29	1.12	0.6288	< 0.001	0.0601
Valerate	3.95[a]	3.65[ab]	2.32[c]	2.52[bc]	0.72	0.0125	< 0.001	0.4876
Isovalerate	1.69	1.67	1.23	1.51	0.38	0.6374	< 0.001	0.4864
Isobutyrate	0.73	0.66	0.58	0.66	0.10	0.5970	< 0.001	0.3328
Total VFA	115.12	124.24	113.43	113.78	9.83	0.7409	< 0.001	0.9484
	moles/100 moles[2]				SEM[1]	Treatment	Time	Treatment x Time
Acetate	49.25	47.70	50.73	48.99	2.13	0.1900	< 0.001	0.9809
Propionate	37.71	39.83	37.68	39.00	2.66	0.6057	< 0.001	1.0000
Butyrate	7.68	7.88	8.01	7.51	0.83	0.9276	< 0.001	0.0010
Valerate	3.27[a]	2.92[ab]	1.99[c]	2.16[bc]	0.50	0.0235	< 0.001	0.7485
Isovalerate	1.69	1.37	1.23	1.51	0.38	0.3875	< 0.001	0.6532
Isobutyrate	0.72	0.58	0.58	0.66	0.10	0.2827	< 0.001	0.8998
Acetate:Propionate	1.38	1.30	1.40	1.34	0.19	0.8597	< 0.001	1.0000

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

[2]moles/100 moles = [VFA (mM)/total VFA (mM)] x 100.

[abc]Least squares means that do not contain at least one of the same letters across rows and columns differ ($P < 0.05$).

Table 3.8. Least square means of nitrogen balance in steers supplemented with Program Nutrition Finisher (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	MT	PN	PN x MT
N Intake, g/d	221.17	219.52	216.28	217.40	9.85	0.4174	0.9506	0.7474
N Fecal Output, g/d	64.28[a]	60.63[ab]	56.43[b]	57.35[b]	2.77	0.0092	0.4846	0.2472
N Urine Output, g/d	83.07	83.44	88.08	74.07	9.13	0.7170	0.2642	0.2401
N Excreted, g/d	147.34	144.07	144.51	131.42	9.28	0.2805	0.2553	0.4897
N Absorbed, g/d	158.51	160.12	160.38	160.77	10.34	0.7845	0.8296	0.8947
N Retained, g/d	73.83	75.45	71.77	85.98	8.44	0.5908	0.3202	0.4270
N Digestibility, %	33.12	34.38	32.58	39.53	3.46	0.4990	0.2347	0.4044

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

[ab]Least squares means that do not contain at least one of the same letters across rows and columns differ ($P < 0.05$).

Table 3.9. Least square means of energy balance in steers supplemented with Program Nutrition Finisher (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	MT	PN	PN x MT
O_2 consumption, L/kg $BW^{0.75}$/d	34.98[b]	39.43[a]	38.69[a]	36.63[b]	0.78	0.4794	0.0722	< 0.001
CO_2 production, L/kg $BW^{0.75}$/d	31.79[c]	36.10[a]	36.21[a]	34.21[b]	0.71	0.0498	0.0702	< 0.001
CH_4 production, L/kg $BW^{0.75}$/d	0.63	0.66	0.65	0.56	0.08	0.4909	0.6089	0.2868
Heat Production, kJ/kg $BW^{0.75}$/d	718.87[c]	812.36[a]	800.55[a]	758.45[b]	15.83	0.2977	0.0627	< 0.001
RQ	0.9092[b]	0.9155[b]	0.9360[a]	0.9340[a]	0.0079	0.0008	0.7061	0.4745
Intake Energy, kJ/kg $BW^{0.75}$/d	1592.12	1593.65	1563.87	1560.99	34.03	0.1321	0.9726	0.9104
Fecal Energy, kJ/kg $BW^{0.75}$/d	334.21	341.39	326.01	304.84	19.33	0.0833	0.5735	0.2606
Digestible Energy, kJ/kg $BW^{0.75}$/d	1257.91	1252.26	1237.86	1256.15	35.60	0.6989	0.7619	0.5676
Digestibility Energy, %	78.97	78.55	79.17	80.46	1.20	0.1891	0.5805	0.2838
Gas Energy, kJ/kg $BW^{0.75}$/d	24.89	26.24	25.81	22.04	3.22	0.4912	0.6091	0.2871
Urine Energy, kJ/kg $BW^{0.75}$/d	37.46	42.41	41.88	26.37	6.44	0.3083	0.3528	0.0811
Metabolizable Energy, kJ/kg $BW^{0.75}$/d	1195.56	1183.62	1170.16	1207.75	37.04	0.9752	0.5333	0.2357
Retained Energy, kJ/kg $BW^{0.75}$/d	476.69	371.26	369.61	449.30	37.98	0.6053	0.6467	0.0035
Energy retained in protein, kJ/kg $BW^{0.75}$/d	111.13	115.98	108.67	129.57	13.59	0.6395	0.2848	0.5011
Energy retained in fat, kJ/kg $BW^{0.75}$/d	365.56	255.27	260.94	319.73	35.83	0.4177	0.3016	0.0026

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

[1]SEM: standard error of the mean, n=8.

[ab]Least squares means that do not contain at least one of the same letters across rows differ ($P < 0.05$).

Table 3.10. Least square means for protein metabolism in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

	Treatment					P-value		
Item	Control	PN	MT	PNMT	SEM[1]	MT	PN	PN x MT
Urea Excretion, g/d	98.67[b]	107.02[ab]	121.33[a]	110.80[ab]	16.25	0.0384	0.8560	0.1281
N Excreted as Urea, g N/d	46.00	50.80	56.57	51.66	7.68	0.0674	0.9858	0.1152
^{15}N Fractional Recovery, g/d	0.2663	0.2575	0.2850	0.3263	0.0362	0.1547	0.5879	0.4071
Protein Turnover, g N/d	175.87	201.95	222.86	165.74	21.90	0.8067	0.4840	0.0715
Protein Synthesis, g N/d	129.87	152.05	166.30	114.09	21.52	0.9718	0.4943	0.1010
Protein Degradation, g N/d	17.94	45.75	62.07	7.23	22.98	0.9021	0.5552	0.0825

Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

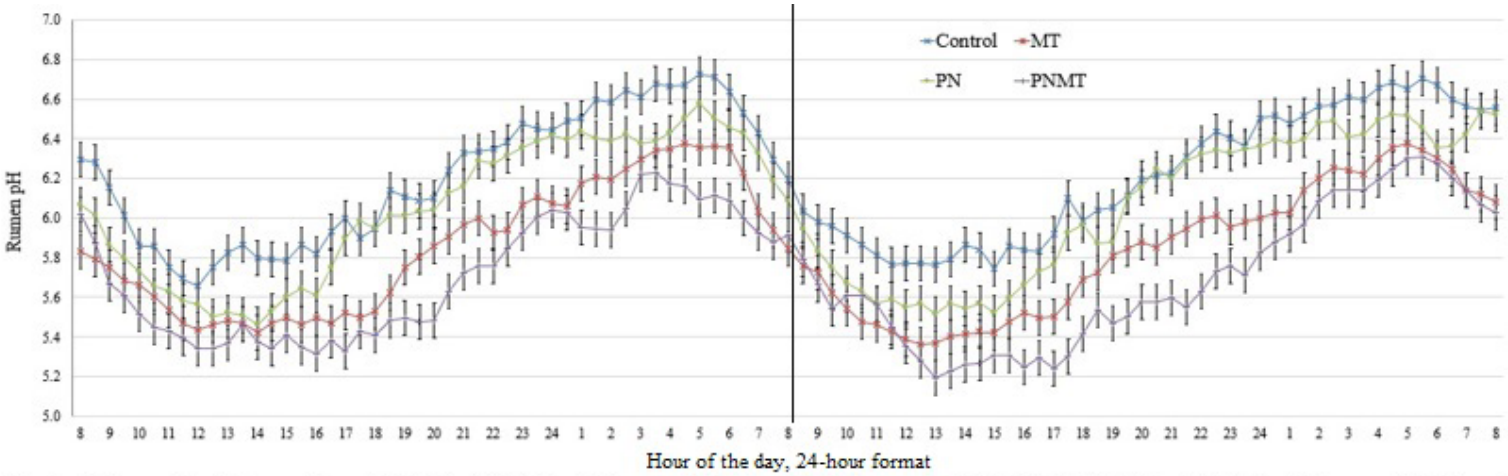
[1]SEM: standard error of the mean, n=8.

[ab]Least squares means that do not contain at least one of the same letters across rows and columns differ ($P < 0.05$).

Table 3.11. Least square means for blood plasma insulin in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

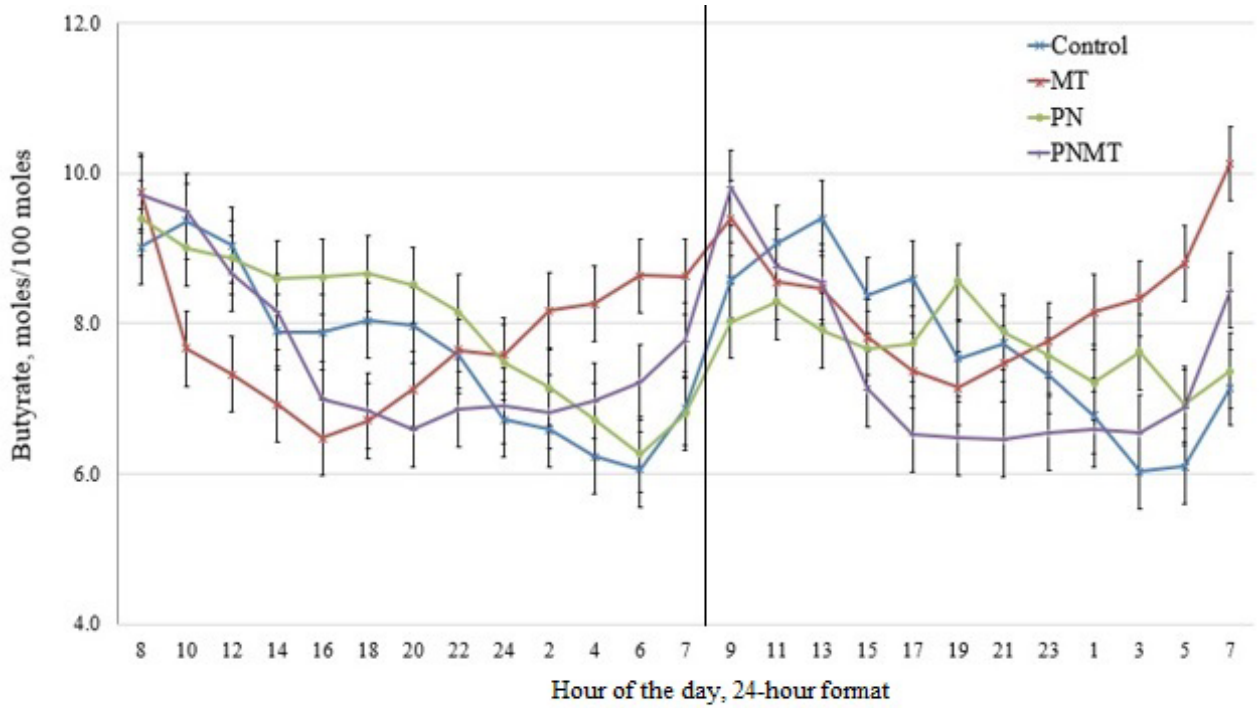
						P-value		
Item	Control	PN	MT	PNMT	SEM[1]	Treatment	Time	Treatment x Time
Insulin, uU/mL	50.02	40.45	38.93	38.90	9.17	0.5845	< 0.001	0.5429

PN = Beef Nutritional Program Finisher; MT = Monensin + Tylosin; PNMT = Beef Nutritional Program Finisher + Monensin + Tylosin
[1]SEM: standard error of the mean, n=8



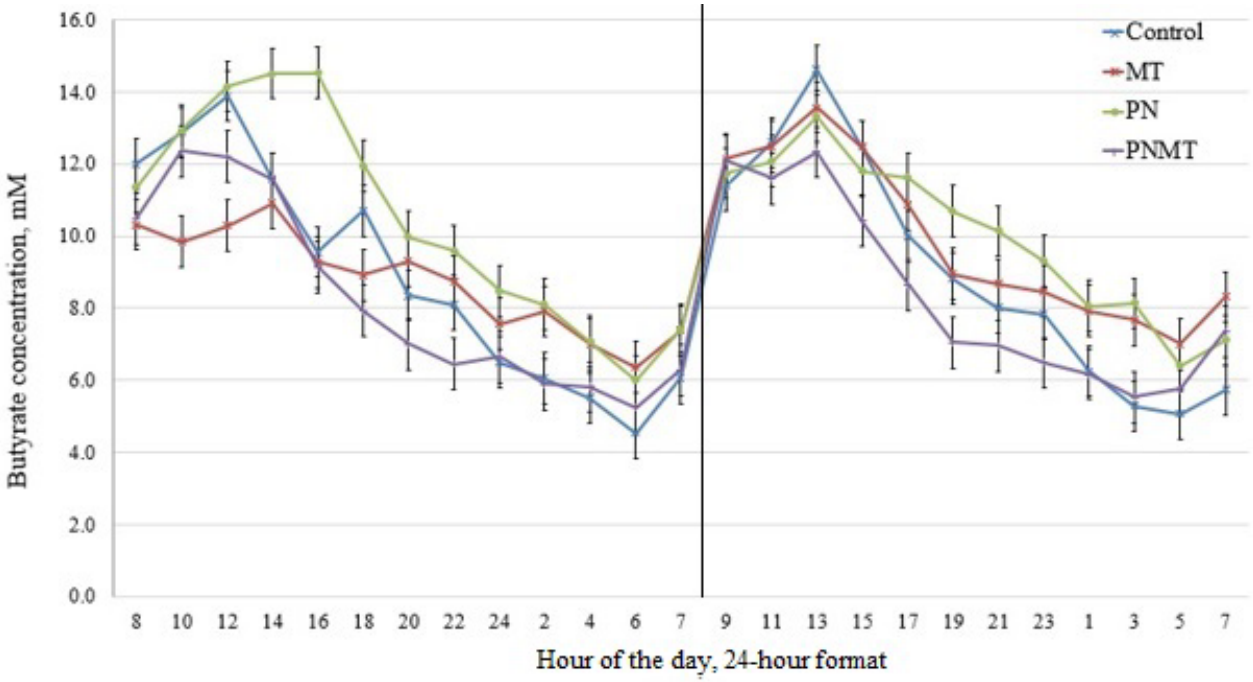
Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

Figure 3.1. Treatment average rumen pH over time in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.



Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

Figure 3.2. Treatment average of ruminal butyrate molar proportion (moles/100 moles) over time in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day



Control: Conventional trace mineral; PN: Beef Nutritional Program Finisher; MT: Monensin + Tylosin; PNMT: Beef Nutritional Program Finisher + Monensin + Tylosin.

Figure 3.3. Treatment average of ruminal butyrate concentration (mM) over time in steers supplemented with Program Nutrition (Beef Nutritional Program Finisher; Alltech Inc., Nicholasville, KY) and Monensin + Tylosin (Elanco Animal Health, Greenfield, IN) at 75 g/head/day.

CHAPTER 4.

In vitro ruminal fermentation patterns of *Aspergillus oryzae* fermentation extract with α-amylase activity

4.1. Introduction

Starch represents a major energy ingredient in many ruminant production systems. Although starch fermentation occurs rapidly in the rumen, its digestion is usually incomplete, leading to the escape of starch to the small intestine (Theurer, 1986). High amounts of starch flowing to the small intestine can result in starch reaching the large intestine, where digestive efficiency is low (Harmon and McLeod, 2001). Thus, improving starch utilization in the rumen and small intestine is crucial when animals are fed high-grain diets. Programmed Nutrition Beef Program (Alltech Inc.) contains *Aspergillus oryzae* fermentation extract which provides α-amylase activity (Amaize, Alltech Inc., Nicholasville, KY) which has the potential to alter gene expression of canonical pathways involved in amino acid and lipid metabolism (Graugnard et al., 2015; Elolimy et al., 2018)

Alpha-Amylase from *Aspergillus oryzae* has an optimum pH between 5.4 and 6.0 and an optimum temperature of 40°C (Klingerman et al., 2009). This enzyme cleaves α-(1,4)-glucosidic linkages present in starch releasing maltodextrin oligosaccharides to non-amylolytic ruminal microorganisms that produce acetate and butyrate (Erratt and Douglas, 1984). The addition of *Aspergillus oryzae* α-amylase can alter ruminal VFA molar proportions by increasing acetate and butyrate while decreasing propionate *in vivo* and *in vitro* (Tricarico et al., 2008). Ruminal starch digestion does not increase with α-amylase

supplementation, but rather it modifies the microbial population and metabolism in the rumen (Tricarico et al., 2005; Tricarico et al., 2008). Rumen fermentation is altered by exogenous α-amylase when feed is ground into smaller (<750 μm) particle sizes (Gallo et al., 2016). Substrate availability is also increased where additional sites for bacterial attachment are exposed, which can lead to an increased feed digestion rate (Gomez-Alarcon et al., 1990; Varel and Kreikemeier, 1994b; Colombatto et al., 2003b).

Supplementation of α-amylase and its effect on average daily gain varies with different diets. Tricarico et al. (2007) observed an increase in average daily gain with *Aspergillus oryzae* α-amylase supplementation in steers consuming steam-flaked corn with cottonseed hulls, cracked corn with corn silage, and high-moisture corn with corn silage. However, no differences in gain were observed when steers consumed steam-flaked corn with alfalfa hay. DiLorenzo et al. (2011) did not observe differences in average daily gain between steers supplemented with α-amylase fed dry-rolled corn or steam-flaked corn. These differences suggest that there are nutrient interactions and differences in microbial populations that impact the ruminal effectiveness of the enzyme.

Barley and wheat are rapidly fermented in the rumen as they are highly digestible starch sources. The endosperm cell wall of these grains presents high levels of β-glucan soluble fiber, a structure that is easily penetrated and digested by cellulolytic bacteria in the rumen (Orskov, 1986; McAllister et al., 1993b). If β-glucans are not completely digested in the rumen they can decrease nutrient absorption in the small intestine, due to changes in intestinal pH and digesta viscosity which affects digestive enzyme activity and gastrointestinal hormone release (Hristov et al., 2000).

Corn and sorghum are not as rapidly fermented in the rumen because their endosperm is composed of a floury region and a horny region. In the floury region, starch associates with protein in a fashion similar to the endosperm of barley and wheat. Meanwhile in the horny region starch is tightly embedded in the protein matrix, making the region resistant to microbial attachment (McAllister et al., 1994; McAllister and Cheng, 1996). Fungi are the only ruminal microorganisms capable of penetrating the horny region of these grains due to the specific activity of its amylases and proteases (McAllister et al., 1993a). Protozoa can impact the rate of starch digestion in the rumen since protozoa can degrade starch by engulfing starch granules, which can take up to 36 h for complete metabolism (Coleman, 1986b). Protozoa can also predate on amylolytic bacteria decreasing the rate of starch digestion (Kurihara et al., 1968).

The effects of *Aspergillus oryzae* fermentation extract supplementation on rumen metabolism and animal performance have been inconsistent across studies. Nisbet and Martin (1990) observed that supplementation with *Aspergillus oryzae* fermentation extract *in vitro* could increase lactate uptake by *Selenomonas ruminantium*, causing an increase in the production of total VFA, and an increase in acetate and propionate molar proportions with a decrease in the acetate:propionate. Tricarico et al. (2005) supplemented different levels of *Aspergillus oryzae* fermentation extract to lactating cows, steers, and in *in vitro* using continuous culture. There were no differences in ruminal total VFA concentrations, but a quadratic increase in acetate:propionate was seen, with an increase of acetate and butyrate and a decrease in propionate molar proportions. Meanwhile, Wiedmeier et al. (1987) fed nonlactating cows chopped alfalfa hay and rolled barley supplemented with

Aspergillus oryzae fermentation extract. No changes in ruminal total VFA concentrations, acetate:propionate, or VFA molar proportions were detected.

The level of *Aspergillus oryzae* fermentation extract supplementation also affects the animal metabolic response. Varel and Kreikemeier (1994b) fed 0, 3, 9, and 27 g/d *Aspergillus oryzae* fermentation extract to nonlactating cows and determined that total VFA concentration increased at the higher levels of supplementation. Tricarico et al. (2005) demonstrated that milk production and blood serum beta-hydroxbutyrate (BHBA) concentration increased when lactating cows were supplemented with α-amylase at 240 DU/kg TMR, but milk production decreased at 480 and 720 DU/kg TMR. Denigan et al. (1992) did not observe any differences in milk yield when supplementing *Aspergillus oryzae* fermentation extract to lactating cows at 0, 1.5, 3, and 6 g/d, but dry matter intake (DMI) was higher at 1.5 g/d. Takiya et al. (2017) also did not detect differences in milk yield when supplementing lactating cows with *Aspergillus oryzae* fermentation extract at 0, 150, 300, or 450 DU/kg DM, but they observed that crude protein digestibility increased linearly with increasing supplementation. In contrast, Oellermann et al. (1990) obtained no differences in crude protein digestibility when supplementing nonlactating cows with *Aspergillus oryzae* fermentation extract at 0, 1, 2, 4, and 6 g/d.

The difference responses reported across studies when supplementing *Aspergillus oryzae* fermentation extract could be due to the different types of substrates present (DiLorenzo et al., 2011), substrate particle size (Gallo et al., 2016), enzyme source (Wallace et al., 2001), enzyme dosage (Tricarico et al., 2005; Tricarico et al., 2008; Takiya et al., 2017), shifts in rumen fluid pH (Klingerman et al., 2009) and animal physiological status (Tricarico et al., 2008). It is possible that Amaize increases acetate and butyrate

ruminal molar proportions by increasing bacterial attachment to feed particles and releasing different maltodextrin oligosaccharides that can be used as a substrate by non-amylolytic microorganisms, which would shift the rumen microbiome dynamics. We hypothesize that the addition of *Aspergillus oryzae* fermentation extract incubated *in vitro* with different substrates results in different profiles of maltodextrin oligosaccharides that can be used by non-amylolytic microorganisms to produce acetate and butyrate at the expense of propionate, and increase total VFA concentration.

We hypothesize that α-amylase (Amaize) supplementation increases *in vitro* starch degradation of different feedstuffs. Retrogradation and flake density of steam-flaked corn could decrease in response to Amaize supplementation due to a higher degree of crystallization and gelatinization of starch, respectively. The objective of this study was to determine the effect of Amaize supplementation on substrate degradation and metabolic parameters impacting feedstuff utilization *in vitro*. Substrates were selected to examine known factors impacting starch utilization such as grain source, degree of processing and retrogradation and to compare these effects on starch availability and gas production, in vitro.

4.2. Material and Methods

4.2.1. Donor animals and diet

Three ruminally cannulated steers were housed in outside pens and fed once a day a diet with 57% rye silage and 34% cracked corn (Table 4.1) *ad libitum*, with no *Aspergillus oryzae* extract α-amylase (Amaize) added.

4.2.2. Assay development

Assay development was accomplished in two phases. In the first phase, we analyzed multiple substrate inclusions without the addition of Amaize to determine the optimal substrate inclusion. The targeted substrate inclusion provided adequate feedstuff to saturate starch hydrolysis without depletion of the starch substrate during the incubation period. Typical substrate amounts employed for in vitro gas production (0.25 g) are chosen so that fermentation reaches a plateau during the incubation and starch is rapidly depleted. Our goal was to develop a system where starch would not be depleted to more effectively study the impact of enzyme addition.

The second phase was composed of a dose-response trial to determine the optimal amount of Amaize. The targeted Amaize dosage must provide enough enzyme to compete for substrate with the microbial enzymes present in the rumen digesta.

Dry-rolled corn was used as the substrate, dried at 55°C for 48 hours, and ground with a Wiley Mill to pass through a 1-mm screen. In all comparisons, Ankom fermentation vessels were run in duplicate per substrate inclusion level and incubation time. Substrate amounts incubated were 0.25, 0.5, 1, 2.5, and 5 g, based on the manufacturer's recommended substrate inclusion of 0.25 g for highly fermentable substrates (Ankom,

2013). The vessels were incubated for 0, 2, 4, 6, 8, 10, 12, or 24-hour, and analyzed for *in vitro* gas production and starch content. *In vitro* gas production was measured using Ankom fermentation vessels with remote automatic pressure transducers (Ankom Wireless Gas Production System, Ankom Technology, Macedon, NY). Starch content analysis was performed at each time point.

Ruminal contents (400 mL) from each steer were collected one hour after feeding, combined, and blended for two minutes under a CO_2 headspace. The blended contents were strained through four layers of cheesecloth into an insulated flask and transported to the laboratory. Rumen buffer, rumen micromineral, rumen macromineral, and reducing solutions were prepared as described by (Goering and Van Soest, 1970b). The rumen buffer solution was prepared by adding 2 g of ammonium bicarbonate and 17.5 g of sodium bicarbonate to 500 mL of water. The rumen micromineral solution was prepared by adding 1.32 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 1 g of manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 0.1 g of cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), and 0.8 g of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) to a 100 mL volumetric flask and adjusting the volume with water. The rumen macromineral solution was prepared by adding 2.85 g of sodium phosphate dibasic (Na_2HPO_4 anhydrous), 3.1 g of potassium phosphate monobasic (KH_2PO_4 anhydrous), and 0.3 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) to 500 mL of water. The reducing solution was prepared by adding, in the following sequence, 1.25 g cysteine•HCl, 8 mL 1 N sodium hydroxide (NaOH), and 1.25 g sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) to a 200 mL volumetric flask and adjusting the volume with water.

The media solution was prepared by adding, in the following sequence, 4 g of trypticase, (BBL™ Typticase™ Peptone pancreatic digest of casein; Becton, Dickson and Company, Sparks, MD), 800 mL of water, 0.2 mL of micromineral solution, 400 mL of buffer solution, 400 mL of micromineral solution, and 2 mL of resazurin (0.1% w/v). The media solution was placed in a 39°C water bath, and a CO_2 hose was inserted into the solution and CO_2 was bubbled slowly. After which, 200 mL of the reducing solution was prepared and added to the media solution. The media solution is reduced once it changes from red color (oxidized) to colorless (reduced). Once the media solution was colorless, 400 mL of filtered rumen fluid was added (total 2200 mL).

Prior to analysis, the volume of each Ankom fermentation vessel (300 mL) was determined by filling it with water and weighing for volume calculations (eq. 1). To each fermentation vessel was added, in the following sequence, the corresponding substrate amount to be tested, 2 mL of water, 100 mL of media solution, followed by 20 seconds of CO_2 gas. The fermentation vessels were then sealed with the remote automatic pressure transducers. Ankom fermentation vessels were incubated in a 39°C water bath, where the gas pressure was measured at 5-minute intervals.

At the end of the incubation period, remote automatic pressure transducers were replaced by caps to seal and each vessel was processed immediately for starch content. Starch content analysis (Knudsen, 1997) with adaptations by Richards et al. (1995) was performed on the contents of the vessels. Briefly, 50 mL of cold 1.2 M acetate buffer containing 0.2% (w/v) benzoic acid was added followed by 400 µL of heat-stable α-amylase (Spezyme Fred, Genencor International, Inc.; Palo Alto, CA). Vessels were capped, mixed, and placed in a 100°C water bath for 1 h. Caps were left slightly loose for

the first 10 min of incubation to release pressure and then tightened for the remaining time of the incubation. Vessels were gently swirled for 10, 30, and 50 min. After incubation, the vessels were cooled in water, and 2 mL of amyloglucosidase (Genencor International, Inc.) were added. Vessels were placed in a 55°C oven for 2 h, gently swirled after 1 h and at the end of the incubation. Once the vessels were cooled, the weight of the vessels with contents and cap were recorded for volume content determination (eq. 5). An aliquot of the solution was then transferred to microcentrifuge tubes and centrifuged at 12,000 x g for 10 minutes at 23°C. The supernatant was analyzed for glucose content using the Konelab 20XTi clinical analyzer and a glucose-hexokinase kit (Thermo Fisher Scientific Inc., Beverly, MA). The standard curve was determined using a standard stock solution (1000 mg/dL glucose), prepared by adding 1 g of oven-dried dextrose, 10 mL of 1% sodium azide, diluted with water to 100 mL. Vessels with no substrate added (reagent blank) were used to calculate free glucose from reagents (eq. 6). Vessels with substrate added but no starch enzyme added (enzyme blank) were used to calculate free glucose (eq. 7). The glucose content of all samples was corrected for free glucose from reagents and substrate (eq. 9), and the starch percentage of each vessel was calculated using the weight of the substrate incubated (eq. 10).

After determining the optimal substrate inclusion, a dose-response trial was performed. The kinetic determination of Amaize α-amylase activity was accomplished to aid dosage determination and ensure enzyme activity throughout incubation periods. Amaize was serially diluted and assayed for α-amylase activity. The α-amylase activity was measured using a reagent set (Teco Diagnostics, Anaheim, CA) adapted to a Synergy HTX multi-mode plate reader pre-heated to 39°C (BioTek, Winooski, VT). The Teco

reagent was reconstituted with 6 mL of water and further diluted with 18 mL of water. The diluted reagent was placed in a 39°C water bath. A blank of saline (5 µL), a control dilution factor to ensure plate repeatability (5 µL), and the multiple dilutions of Amaize (5 µL) were added to a 96-well plate. After samples were loaded, 200 µL of heated (39°C) Teco reagent was added using a multichannel pipette. The α-amylase activity was measured every 30 seconds for 15 minutes (eq. 13).

After determining the α-amylase activity, multiple Amaize dosages were incubated in Ankom fermentation vessels with substrate and rumen inoculum. The substrate used was dry-rolled corn, dried at 55°C for 48 hours and ground with a Wiley Mill to pass through a 1-mm screen. The Amaize dosages included 0, 10, 15, and 20 IU/100 mL inoculum. Amaize was pipetted into the Ankom fermentation vessels after substrate adding, followed by adding inoculum. The ruminal contents and media solution were prepared as described previously, adjusted to the required final volume. To each Ankom fermentation vessel was added 2.5 g of substrate, 2 mL of water containing the corresponding Amaize dosage level (blanks received 2 mL of water without Amaize added), and 100 mL of inoculum, was added to each Ankom fermentation vessel which was then followed by 20 seconds of CO_2 gas and sealed with screw caps. Glucose content from the supplemental enzyme was accounted for by including a fermentation jar without substrate added for each Amaize dosage (Amaize blank). The fermentation jars were incubated for 0, 2, 8, and 10 hours. To ensure enzyme activity was detected throughout incubation times, a 5 µL aliquot was obtained from the fermentation vessels at the end of each incubation period and analyzed for amylase activity as previously described.

To further test the selected dosage level and optimal fermentation time, amylopectin (1 g and 1.8 g) and dry rolled corn (1.5 g and 2.5 g) were incubated with Amaize at 0 or 15 IU/100 mL inoculum for 0, 1, 2, 4, 6, 8, 10, and 16-hour across multiple runs.

4.2.3. *In vitro* gas production and starch degradation of different feedstuffs

Experiment 1

Dry rolled barley, Steam-flaked barley, Corn steam-flaked at 26 and 32 lb/bushel

Corn steam-flaked at 26 lb/bu (335 g/L), or 32 lb/bu (412 g/L), dry rolled barley (438 g/L), and steam-flaked barley (357 g/L) were used as substrates. All substrates were dried for 48 hours in a 55°C oven, and ground with a Wiley Mill to pass through a 1-mm screen.

Starch Content and Availability

Total starch, available starch, and starch availability were determined as described by Xiong et al. (1990b) with adaptations by Hall (2009). These measurements were replicated on three separate runs performed on the same day.

Available Starch

Available starch was determined by adding 0.4 g of substrate, 30 mL of acetate buffer solution (4.1 g/L anhydrous sodium acetate; 4.1 mL/L glacial acetic acid; pH = 4.50), and 1 mL of amyloglucosidase working solution (200 U/mL; AMIGASE GA 400L; Centerchem, Inc., Norwalk, CT) to 50-mL glass vials with screw tops. Available starch-free glucose (amyloglucosidase blanks) were used to account for free glucose present in the substrates. Available starch-free glucose was determined by adding 0.4 g of substrate and 30 mL of acetate buffer solution (4.1 g/L anhydrous sodium acetate; 4.1 mL/L glacial acetic acid; pH = 4.50) to 50-mL glass vials with screw tops. No amyloglucosidase working solution was added to available starch-free glucose vials. Available starch and available starch-free glucose vials were capped, mixed, and placed in a 40°C water bath for 60 minutes. The vials were shaken vigorously during incubation at 10, 20, 30, 40, 50, and 60 minutes. The vials were removed from the water bath then, 4 mL of zinc sulfate solution (10% (w/v); 100 g/L zinc sulfate heptahydrate $ZnSO_4 \cdot 7\ H_2O$) and 2 mL of sodium hydroxide solution (0.5 N; 20 g/L sodium hydroxide) were immediately added to the vials, followed by dilution to a final volume of 50 mL with water. Then, the vials were capped and mixed. Standard glucose solution was prepared at 7.8125, 15.625, 31.25, 62.5, 125, 250, and 500 mg/dL using a standard stock solution (1000 mg/dL glucose). A blank saline, standard glucose solution and samples were loaded (5 µL) to a 96-well plate in replicate. Next, 250 µL of glucose hexokinase liquid stable reagent (Infinity™ Glucose Hexokinase; Fisher Diagnostics, LLC; Middletown, VA) was added to all wells. Glucose concentration was measured using a Synergy HTX multi-mode plate reader pre-heated to 39°C (BioTek, Winooski, VT) read at 340 nm. Starch availability was calculated as described (eq. 14).

Total Starch

Total starch was determined by adding 0.2 g of substrate and 30 mL of acetate buffer solution to 50-mL glass vials with screw tops. Total starch-free glucose (amyloglucosidase blanks) was used to account for free glucose present in the substrates. Total starch-free glucose was determined by adding 0.2 g of substrate and 30 mL of acetate buffer solution to 50-mL glass vials with screw tops. Total starch and total starch-free glucose vials were capped and placed in a 94°C water bath for 75 minutes, hand-swirled every 15 minutes. The vials were removed from the water bath, cooled in running water for 15 minutes. Once vials were cooled, 1 mL of amyloglucosidase working solution (200 U/mL) was added to total starch vials only. No amyloglucosidase working solution was added to total starch-free glucose vials. All vials were capped, mixed, and placed in a 40°C water bath for 60 minutes. The vials were vigorously shaken every 10 minutes during incubation. The vials were removed from the water bath then, 4 mL of zinc sulfate solution (10% (w/v) and 2 mL of sodium hydroxide solution (0.5 N) were immediately added to the vials followed by dilution to a final volume of 50 mL with water. Then, the vials were capped and mixed. Standard glucose solution was prepared as previously described above. A blank saline, standard glucose solution and samples were loaded (5 µL) to a 96-well plate in replicate. Next, 250 µL of glucose hexokinase liquid stable reagent (Infinity™ Glucose Hexokinase) was added to all wells. Glucose concentration was measured using a Synergy HTX multi-mode plate reader pre-heated to 39°C (BioTek, Winooski, VT) read at 340 nm. Total starch values were used to calculate starch availability as described (eq. 14).

Gas Production

I*n vitro* gas production was measured for 24 hours using Ankom fermentation vessels with remote automatic pressure transducers as previously described. These measurements were replicated on five separate days. To each fermentation vessel was added 2.5 g of ground substrate, 1.5 mL of water containing the corresponding Amaize dosage level (0 IU or 15 IU/100 mL inoculum), and 100 mL of inoculum, followed by 20 seconds of CO_2 gas. Vessels were sealed with remote automatic pressure transducers (Ankom Wireless Gas Production System, Ankom Technology, Macedon, NY) and incubated in a 39°C water bath for 24 hours. Two-hour gas production was calculated using gas produced at the 2 hour incubation time point. Ruminal contents and media solution were prepared as described previously, and adjusted for the final volume.

Starch Degradation

For each incubation the percent starch degraded after 2-hour incubation was measured. At the end of the 2-hour incubation time, 50 mL of cold 1.2 M acetate buffer in 0.2% (w/v) benzoic acid was added. Starch analysis was performed as previously described above. Starch remaining in the vessel was calculated (eq. 11) and these values were used to calculate the percent starch degraded (eq. 12).

Experiment 2

Dry Rolled Corn, High moisture Corn and Corn steam-flaked at 27 lb/bushel

Dry rolled corn, high moisture corn, and corn steam-flaked at 27 lb/bu (347 g/L) were used as substrates. All substrates were dried for 48 hours in a 55°C oven, and ground with a Wiley Mill to pass through a 1-mm screen.

Starch Content and Availability

Total starch and starch availability of substrates were determined as previously described above.

Gas Production

Gas production was measured for 24 hours using Ankom fermentation vessels with remote automatic pressure transducers as previously described for five separate days. Ruminal contents and media solutions were prepared as described previously. Substrates were incubated with Amaize at 0 or 15 IU/100 mL.

Starch Degradation

Starch degradation was measured in 100 mL-glass culture tubes with PTFE lined screw caps (32 mm x 200 mm, Aldrich®, Sigma-Aldrich Co.). To each culture tube was

added 0.625 g of ground substrate, 0.375 mL of water containing the corresponding Amaize dosage level (0 IU or 15 IU/100 mL inoculum), and 25 mL of inoculum, followed by 20 seconds of CO_2 gas. Tubes were capped and incubated in a 39°C water bath for 2 hours. At the end of the 2-hour incubation time, 12.5 mL of cold 1.2 M acetate buffer in 0.2% (w/v) benzoic acid was added. Starch analysis was performed as previously described above. Starch remaining in the vessel was calculated (eq. 11) and these values were used to calculate starch degraded after incubation (eq. 12).

Experiment 3

Corn steam-flaked at 23, 25, 27, 29, and 31 lb/bushel with or without retrogradation

Steam-flaked corn and retrograde steam-flaked corn with five different flake densities were used as substrates. Flake densities of both retrograde and non-retrograde steam-flaked corn were 23 lb/bu (296 g/L), 25 lb/bu (322 g/L), 27 lb/bu (347 g/L), 29 lb/bu (373 g/L), and 31 lb/bu (399 g/L). Retrogradation was achieved by exposing steam-flaked corn to 55°C in heat-sealed foil bags, as described by Trotta et al. (2021a). All substrates were dried for 48 hours in a 55°C oven, and ground with a Wiley Mill to pass through a 1-mm screen.

Starch Content and Availability

Total starch and starch availability of substrates were determined as described previously.

Gas Production

Gas production was measured for 24 hours using Ankom fermentation vessels with remote automatic pressure transducers as previously described for five separate days. Ruminal contents and media solutions were prepared as described previously. Substrates were incubated with Amaize at 0 or 15 IU/100 mL.

Starch Degradation

Starch degradation was measured in 100 mL-glass culture tubes with PTFE lined screw caps (32 mm x 200 mm, Aldrich®, Sigma-Aldrich Co.) as described previously. Substrates were incubated with Amaize at 0 or 15 IU/100 mL.

4.2.4. Calculations

***In Vitro* Gas Production**

The Ankom pressure transducer system measures pressure (psi) changes from baseline. Cumulative gas pressure measured was converted to moles of gas produced at

standard pressure and temperature using the ideal gas law (eq. 1). Moles of gas produced was converted to mL of gas produced Avogadro's law, where at atmospheric pressure (1 psi = 6.894757293 kPa) 1 mole occupies 22.4 L at 0°C, so 1 mole occupies 25.6 L at 39°C (312°K) (eq. 2). Both concepts were used to calculate the volume of gas produced (eq. 3). Data were fit into multiple models described by Pitt et al. (1999) using MatLab (MatLab Software Version R2021b, The MathWorks, Inc., Natick, MA) for the best-fit determination. Nonlinear gas production models tested were Cone, Fitzhugh, Gompertz, Weibull, France, Richards, Cauchy, Exponential, Logistic and Feller. Cone function presented the highest coefficient of determination (r^2) and lowest root mean square error (RMSE). Data were analyzed using the Cone cumulative gas production function (eq. 4) described by Pitt et al. (1999) with the assumption that no gas is produced until a discrete-time lag has elapsed. Accumulated pressure data was used to calculate the rate, lag time, total gas produced, and plateau with the application of the Cone model using MatLab (Pitt et al., 1999)

Ideal gas law (eq. 1)

- $V = (V_{vessel} - Wgt_{contents}) / 1000$
- $n = p (V/ RT)$

V : headspace volume in fermentation vessel (L)

V_{vessel} : weight of each fermentation vessel full of water (mL)

$Wgt_{contents}$: weight of contents added to each fermentation vessel (g)

n : gas produced (moles)

p : pressure (kPa)

T : temperature (K)

R : gas constants (8.314472 L kPa/K/mol)

Avogrado's law (eq. 2)

- gas produced (mL) = n * 25.6 * 1000

n : gas produced (moles)

Volume of gas produced (eq. 3)

- $P = (P_{atm} / 14.696) * 101.325$
- $\Delta V = (\Delta P * V / P) * 1000$

P : atmospheric pressure in kPa adjusted to STP (101.325 kPa = 14.696 psi at STP)

P_{atm} : average of the background atmospheric pressure (in)

ΔV : gas produced at standard atmospheric pressure (mL)

ΔP : plateau pressure produced during fermentation (kPa)

V : headspace volume in fermentation vessel (L)

Cone cumulative gas production function (eq. 4)

- $F(t) = 1 / (1 + (r * t)^{(-n)})$

r : rate constant

t : time (h)

n : discrete time lag (h)

- Rate of Degradation (%/h) = k * 100

k: rate of degradation

Volume remaining in vessel after starch analysis (eq. 5)

- Each vessel contained a different ΔV_{final}, which was used to calculate glucose and starch content of the corresponding substrate contained in the vessel.
- $\Delta V_{final} = ((W_f - W_i) / 0.998) / 100$

ΔV_{final} : volume remaining in vessel after starch analysis (dL)

W_f : weight vessel with contents and cap after starch analysis (g)

W_i : weight vessel with substrate, media solution and cap before *in vitro* incubation (g)

Starch Degradation

Free glucose from reagent blank (eq. 6)

- $Glucose_{reagent} = GC_{reagent} * \Delta V_{final}$

$Glucose_{reagent}$: free glucose from reagent blank (mg)

$GC_{reagent}$: glucose concentration in reagent blank (mg/dL)

ΔV_{final} : volume remaining in vessel after starch analysis (dL)

Free glucose from enzyme blank (eq. 7)

- $Glucose_{substrate} = GC_{substrate} * \Delta V_{final}$

$Glucose_{substrate}$: free glucose from enzyme blank (mg)

$GC_{substrate}$: glucose concentration in enzyme blank (mg/dL)

ΔV_{final} : volume remaining in vessel after starch analysis (dL)

Glucose content from Amaize (eq.8)

- $Glucose_{Amaize} = (GC_{Amaize} * \Delta V_{final}) - Glucose_{reagent}$

$Glucose_{Amaize}$: glucose content from Amaize blank (mg)

GC_{Amaize}: glucose concentration in Amaize blank (mg/dL)

ΔV_{final} : volume remaining in vessel after starch analysis (dL)

$Glucose_{reagent}$: free glucose from reagent blank (mg)

Amount of glucose in sample (eq. 9)

- $Glucose_{sample} = (GC_{sample} * \Delta V_{final}) - Glucose_{reagent} - (GC_{substrate} * Wgt_{substrate})$
- $Glucose_{sample} = (GC_{sample} * \Delta V_{final}) - Glucose_{reagent} - (GC_{substrate} * Wgt_{substrate}) - Glucose_{Amaize}$

$Glucose_{sample}$: glucose content in sample (mg)

GC_{sample}: glucose concentration in sample (mg/dL)

ΔV_{final} : volume remaining in vessel after starch analysis (dL)

$Glucose_{reagent}$: free glucose from reagent blank (mg)

$Glucose_{substrate}$: free glucose from starch enzymes (mg)

$Wgt_{substrate}$: weight of substrate added to vessel prior to *in vitro* gas production (mg)

$Glucose_{Amaize}$: glucose content from Amaize blank (mg)

Starch percentage in sample (eq. 10)

- % Starch = $[(Glucose_{sample} / 1.110) / (Wgt_{substrate} * DM)] * 100$

% Starch : sample starch content on a dry-matter basis (%, DM)

$Glucose_{sample}$: glucose content in sample (mg)

1.110 : correction factor for glucose recovery from starch

$Wgt_{substrate}$: weight of substrate added to vessel prior to *in vitro* gas production (mg)

DM: Dry Matter (%)

Starch remaining in Vessel (eq. 11)

- Starch in vessel (mg) = (% Starch / 100) * $Wgt_{substrate}$

Starch in vessel (mg): starch content in the vessel at incubation time

% Starch : sample starch content on a dry-matter basis (%, DM)

$Wgt_{substrate}$: weight of substrate added to vessel prior to in vitro gas production (mg)

Starch degraded after incubation (eq. 12)

- Starch degraded (mg) = Starch in vessel at hour 0 – Starch in vessel at hour 2

Starch in vessel at hour 0 (mg): starch content in vessel at 0 hour incubation time

Starch in vessel at hour 0 (mg): starch content in vessel at 2 hour incubation time

Biotek Plate Reader

Amylase Activity (IU/mL) (eq. 13)

- Amylase Activity (IU/mL) = [[(Max V / 1000)*0.205]/[(8.5*0.005*0.6018)]]*DF

Max V: plate reader output

DF: Dilution Factor Amaize

Available starch, total starch, and starch availability (eq. 14)

- Available starch free-glucose, % = 100 * [(G_{AFG} * V_{AFG} * 0.9) / (Wt_{AFG} * 1000)]

G_{AFG}: Glucose concentration available starch-free glucose (g/L)

V_{AFG}: Final solution volume available starch-free glucose (50 mL)

Wt_{AFG}: weight sample available starch-free glucose (g)

- Available starch, % = [100 * [(G_A * V_A * 0.9) / (Wt_A * 1000)]] - FG_A

G_A: Glucose concentration available starch (g/L)

V_A: Final solution volume available starch (50 mL)

Wt_A: weight sample available starch (g)

FG_A: available strach-free glucose (%)

- Total starch free-glucose, % = 100 * [(G_{TFG} * V_{TFG} * 0.9) / (Wt_{TFG} * 1000)]

G_{TFG}: Glucose concentration total starch-free glucose (g/L)

V_{TFG}: Final solution volume total starch-free glucose (50 mL)

Wt_{TFG}: weight sample total starch-free glucose (g)

- Total starch, % = [100 * [(G_T * V_T * 0.9) / (Wt_T * 1000)]] – FG_T

G_T: Glucose concentration total starch (g/L)

V_T: Final solution volume total starch (50 mL)

Wt_T: weight sample total starch (g)

FG_T: total starch-free glucose (%)

- Starch availability, % = 100 * (AS / TS)

AS: Available starch

TS: Total starch

4.2.5. Statistical Analysis

The experimental model used for *in vitro* gas production parameters and starch degraded in experiments 1, 2 and 3 was a randomized complete block design, where blocks represented 5 separate days. The data were analyzed using the GLM procedure of SAS (SAS version 9.4; SAS Inst. Inc., Cary, NC). Mean differences were compared using least significant difference (LSD) and Tukey test features of SAS. Treatments and interactions

were considered different when $P < 0.05$ and a trend when $P < 0.10$. Experiment 1 compared the effects of the substrates corn steam-flaked at 26 lb/bu, corn steam-flaked at 32 lb/bu, dry rolled barley, and steam-flaked barley with and without Amaize supplementation. Experiment 2 analyzed the effects of different corn types (dry rolled corn, high moisture corn, and corn steam-flaked at 27 lb/bu) with or without Amaize supplementation. Experiment 3 analyzed the effects of different steam-flaked corn flake densities (23, 25, 27, 29, and 31 lb/bu) and the effect of retrogradation, with or without Amaize supplementation.

4.3. Results

4.3.1. Assay development

It was determined that 2, 4, 6, 8, 10, and 12-hour incubation times did not provide adequate time to reach a gas production plateau (data not shown). Substrate inclusion at 0.25 and 0.5 g was rapidly fermented, producing inconsistent starch content results (data not shown). The results of this assay demonstrated that a substrate inclusion at 2.5 g generated the most consistent linear decrease in starch content over time (data not shown). Substrate inclusion at 2.5 g allows detection of fermentation pattern changes before a maximum response is achieved without complete substrate depletion (data not shown).

On average, there was a 4.07 % increase in starch remaining in the vessel between Amaize at 0 and 15 IU/100 mL inoculum after 8, 10, and 16-hour incubations (data not

shown). The greatest increase in starch content resulting from Amaize addition was detected at the 2-hour incubation time, with an average of a 12.20% increase in starch disappearance using 2.5 g of dry rolled corn as substrate (data not shown).

Amaize α-amylase activity diluted at 0.8, 1.6, and 4 IU/mL did not produce a linear response in starch hydrolysis, indicating that the enzyme concentration was too low for accurate measurements. Amaize amylase activity diluted at 0.2, 0.27, 0.32, and 0.4 IU/mL generated a linear response and averaged at 400 IU/mL (data not shown).

This assay determined that Amaize added at 15 IU/100 mL inoculum incubated with 2.5 g of substrate for 2 hours produced the greatest increase in percent starch degradation (data not shown). We felt this would be the most sensitive combination to study its impact on starch degradation. These measures in combination with 24 hour fermentations allows the determination of gas production to estimation both kinetics and the extent of digestion. These conditions were selected to study the *in vitro* gas production and starch degradation of different feedstuffs (experiments 1, 2 and 3).

4.3.2. *In vitro* gas production and starch degradation of different feedstuffs

Experiment 1

Dry rolled barley, Steam-flaked barley, Corn steam-flaked at 26 and 32 lb/bushel

Starch availability was lower for dry rolled barley, followed by steam-flaked barley, corn steam-flaked at 26 lb/bushel, and corn steam-flaked at 32 lb/bushel (Table 4.2). *In vitro* gas production approached plateau faster for dry rolled barley than for steam-flaked barley, corn steam-flaked at 26 lb/bushel, and corn steam-flaked at 32 lb/bushel (Figure 4.1).

Although no differences in gas production after 2-hour incubation were detected, the percent starch degraded at 2 h was increased 1.5% and 3.5% by Amaize addition to corn steam-flaked at 26 and 32 lb/bu whereas Amaize did not increase starch degradation for barley (Substrate x Amaize; $P = 0.0002$) (Table 4.3).

There was a decrease in gas production after 24-hour when Amaize was added for steam-flaked barley, but there was an increase in gas production for dry rolled barley when Amaize was present (Substrate x Amaize; $P < 0.001$) (Table 4.3). The inclusion of Amaize did not change *in vitro* gas production after 24-hour for corn steam-flaked at 26 and 32 lb/bushel (Substrate x Amaize; $P < 0.001$) (Table 4.3). Amaize inclusion increased the rate of degradation for corn steam-flaked at 26 lb/bushel and steam-flaked barley (Substrate x Amaize; $P < 0.001$) (Table 4.3). No differences in the rate of degradation were detected when Amaize was incubated with corn steam-flaked at 32 lb/bushel and dry-rolled barley (Substrate x Amaize; $P < 0.001$) (Table 4.3).

Experiment 2

Dry Rolled Corn, High moisture Corn and Corn steam-flaked at 27 lb/bushel

Starch availability was lower for dry rolled corn, followed by high moisture corn and corn steam-flaked at 27 lb/bushel (Table 4.2).

While curves appear visually similar (Figure 4.2), gas production after 24 hours was significantly higher for high moisture corn than corn steam-flaked at 27 lb/bu and dry rolled corn (Corn type x Amaize; P = 0.0297) (Table 4.4). The effect of different grain processing and α-amylase supplementation can be observed in the gas production curves of dry rolled corn, high moisture corn, and corn steam-flaked at 27 lb/bushel incubated with and without Amaize for 24 hours (Figure 4.2). The curves demonstrate that among the three substrates, high moisture corn generates the most gas over the 24 hour incubation period, while dry rolled corn produces the least (Figure 4.2).

There were no differences in the gas produced after 2-hour incubation between the three substrates (Corn type; P = 0.8454) (Table 4.4); however, the starch degraded after a 2-hour incubation was higher for high moisture corn than dry rolled corn (Corn type; P = 0.0123) (Figure 4.3) (Table 4.4). Dry rolled corn had a higher rate of degradation when Amaize was included (Corn type x Amaize; P < 0.001) (Table 4.4). Amaize inclusion decreased the rate of degradation when corn steam-flaked at 27 lb/bushel was the substrate (corn type x Amaize; P < 0.001) (Table 4.4).

Experiment 3

Corn steam-flaked at 23, 25, 27, 29, and 31 lb/bushel with or without retrogradation

Enzymatic starch availability decreased with retrogradation and as flake density increased, being highest for corn steam-flaked at 23 lb/bu and lowest for retrograded corn steam-flaked at 31 lb/bu (Table 4.2).

Due to the retrogradation effect, retrograded steam-flaked corn took longer to reach a gas production plateau than non-retrograded steam-flaked corn across all flake densities (Figure 4.4). Non-retrograded steam-flaked corn appears to achieve plateau earlier in the incubation than their corresponding retrograded flake densities (Figure 4.4). Although retrograded steam-flaked corn might take longer to reach a plateau, it appears that gas production of retrograded steam-flaked corn surpasses that of non-retrograded steam-flaked corn towards the end of the 24 hour incubation period (Figure 4.4). Amaize inclusion appears to increase gas production of retrograded steam-flaked corn throughout the 24 incubation period, with a higher impact on retrograded corn steam-flaked at 25 lb/bu (Figure 4.4).

Flake density quartically affected *in vitro* gas production after 24- (quartic; P = 0.0109) and 2-hour (quartic; P = 0.0007) incubations, and the rate of degradation (quartic; P = 0.0004) (Table 4.5). Corn steam-flaked at 27 lb/bu presented numerically lower gas production after 2- (quartic; P = 0.0007) and 24- hour (quartic; P = 0.0109) incubations than any other flake density (Table 4.5). Gas production at 2-hour was numerically higher for corn steam-flaked at 23 lb/bu, followed by corn steam-flaked at 25 lb/bu, corn steam-flaked at 29 lb/bu, and corn steam-flaked at 31 lb/bu (quartic; P = 0.0007) (Table 4.5). Gas production after 24-hour was highest for corn steam-flaked at 23 lb/bu, followed by corn steam-flaked at 31 lb/bu, corn steam-flaked at 29 lb/bu, and corn steam-flaked at 25 lb/bu (quartic; P = 0.0109) (Table 4.5). Starch degraded after a 2-hour incubation was highest

for corn steam-flaked at 23 lb/bu and numerically decreased as flake density increased, with the exception of corn steam-flaked at 27 lb/bu which presented the lowest percent starch degradation (quadratic; $P < 0.001$) (Table 4.5). The rate of degradation behaved in a unique manner, where it was highest for corn steam-flaked at 27 lb/bu, followed by corn steam-flaked at 25 lb/bu, corn steam-flaked at 29 lb/bu, corn steam-flaked at 23 lb/bu, and corn steam-flaked at 31 lb/bu (quartic; $P = 0.0004$) (Table 4.5).

The impact of retrogradation could be seen in the gas production measures collected at 2 and 24 h (Table 4.6). Steam-flaked corn produced greater amounts of gas after a 2-hour incubation than retrograded steam-flaked corn without Amaize supplementation (Retrogradation x Amaize; $P = 0.0383$) (Table 4.6). Amaize inclusion increased gas production at 2-hour for retrograded steam-flaked corn but did not affect non-retrograded steam-flaked corn (Retrogradation x Amaize; $P = 0.0383$) (Table 4.6). Steam-flaked corn had higher starch degradation than retrograded steam-flaked corn, where Amaize supplementation improved percent starch degradation for both retrograde and non-retrograde steam-flaked corn (Retrogradation x Amaize; $P = 0.0138$) (Table 4.6). Gas production after 24-hour was higher for retrograded steam-flaked corn than non-retrograded steam-flaked corn, with or without Amaize supplementation (Retrogradation x Amaize; $P = 0.0003$) (Table 4.6). Although Amaize increased gas production after the 24-hour incubation for retrograded steam-flaked corn, it did not improve gas production after 24-hour for non-retrograded steam-flaked corn (Retrogradation x Amaize; $P = 0.0003$) (Table 4.6). The rate of degradation was higher for non-retrograded steam-flaked corn than retrograded steam-flaked corn (Retrogradation x Amaize; $P = < 0.001$) (Table 4.6). Amaize inclusion increased the rate of degradation for retrograded steam-flaked corn but did not

affect it for non-retrograded steam-flaked corn (Retrogradation x Amaize; $P < 0.001$) (Table 4.6).

4.4. Discussion

Cereal grains used in feedlot diets can vary in ruminal and post-ruminal digestibility, where grain processing can improve these parameters (Huntington, 1997). Feedlot finishing diets use steam-flaked corn most often due to its higher starch availability and rate of ruminal starch fermentation (Zinn et al., 2002). Flake density is determined by the separation distance of the rolls when the corn is processed, where the most commonly recommended flake density is 27 lb/bu (Vasconcelos and Galyean, 2007). Steam-flaked grains can retrograde when exposed to high temperatures above 55°C. The elevated temperature causes retrogradation because of the reassociation of dispersed starch molecules. This generates a reduction in porosity of the starch matrix, which reduces starch availability and limits enzyme access (Zinn et al., 2002; McAllister et al., 2006). The degree of starch gelatinization is commonly measured using enzymatic starch availability procedures, where high levels of starch availability can indicate rapid ruminal starch fermentation rates (Vasconcelos and Galyean, 2007; Schwandt et al., 2016).

In vitro gas production can be used to study feedstuff digestibility, where *in vitro* gas production generally increases as substrate disappearance increases, indicating a positive relationship between feedstuff degradation and *in vitro* gas production (Theodorou et al., 1994). During assay development, our data indicated that the effects of Amaize likely occur early during the incubation. In this study, starch degradation of different feedstuffs

was measured after a 2-hour incubation period; since we observed the highest impact of Amaize supplementation on starch degradation at this time point.

Barley is slowly colonized and digested by ruminal bacteria due to its cuticular outer surface of the husk (Cheng et al., 1980; Cheng et al., 1981; Huntington, 1997). Cracks present in the husk allow microbial access to the inner surface where more digestible nutrients are present, permitting microbial proliferation and biofilm development (Cheng et al., 1981; Cheng et al., 1983/84; Rode et al., 1986). By processing grains via dry rolling or steam-flaking, microbes can access and digest starch granules within the endosperm at faster rates (Cheng et al., 1973; Theurer, 1986; McAllister et al., 1990; Offner et al., 2003; Hoffman et al., 2012). In this study, we observed that steam-flaked barley without Amaize supplementation generates higher *in vitro* gas production after 24-hour and starch degradation at 2 h than dry rolled barley, corn steam-flaked at 26 lb/bu and corn steam-flaked at 32 lb/bu. In our data, the gas production curve for steam-flaked barley visually appears to be linear with no apparent plateau. However, there was no overestimation of the data during plateau prediction in that the last point recorded at 24-hour incubation was similar to the estimated plateau. Mathison et al. (1991) reported similar results to our study, where the rate of starch degradation was higher in steam-flaked barley than dry-rolled corn. Trei et al. (1970) reported that *in vitro* gas production increased when barley was steam-flaked, producing more gas than untreated barley. Our study results agree with the findings of Zinn (1993), who reported that ruminal organic matter and starch digestibility were greater for steam-rolled barley, followed by steam-flaked corn and dry-rolled barley. The improvements in *in vitro* gas production and starch degradation with steam-flaking observed in our study could be a result of grain processing, generating higher microbial

access to starch granules and increasing enzyme accessibility with starch gelatinization (Galyean et al., 1976; Galyean et al., 1981; Nocek and Tamminga, 1991; Beauchemin et al., 1994; Mathison, 1996). In this study, the rate of degradation was highest for dry rolled barley and lowest for steam-flaked barley, which could have been a result of more rapidly fermented fine particles (< 1 mm) present in dry-rolled barley (Engstrom et al., 1992).

The hydrolytic capacity of α-amylase is correlated to the physical state of starch, where its effect on native starch is limited (Walker and Hope, 1963). In this study, there was an increase in *in vitro* gas production after 24-hour incubation with no changes in the rate of degradation when dry rolled barley was incubated with Amaize. Meanwhile, there was a decrease in *in vitro* gas production after 24-hour incubation with an increase in the rate of degradation when Amaize was incubated with steam-flaked barley. Enzymatic accessibility to starch granules increases when starch is exposed to temperatures between 55°C to 60°C, due to degradation of the outer layer and reduction in the crystallization of starch granules (Lauro et al., 1963). Dry rolling is a cold processing method, while steam-flaking exposes the grain to moisture and heat to gelatinize the starch (Van Soest, 1994). This study observed an increased rate of degradation when Amaize was incubated with steam-flaked barley and corn steam-flaked at 26 lb/bu, which could result from higher enzymatic access of the less crystallized starch granule. The lack of change in the rate of degradation for corn steam-flaked at 32 lb/bu and dry rolled barley could be due to the less available starch present in dry rolled and high flake density steam-flaked corn. Our data demonstrates that Amaize has the potential to improve starch degradation of corn steam-flaked at 26 and at 32 lb/bu. Chai et al. (2004) demonstrated that gas production could be used to accurately estimate starch degradation once fermentation of starch and other non-

soluble components occurs simultaneously. It is important to emphasize that in our study the effect of Amaize was detected at 2-hour, in which the impact of starch degradation and Amaize supplementation *in vivo* may depend on ruminal retention time and passage rate.

Xiong et al. (1990a) used reconstituted steam-flake sorghum with five different flake densities to compare enzymatic glucose release and *in vitro* gas production to estimate ruminal starch availability; the study determined that both methods can be used as a quantitative measurement of grain processing effects on ruminal starch availability. Our data shows that starch availability does not always reflect starch degradation after 2-hour incubation for different feedstuffs. In our study, starch availability was numerically higher for corn steam-flaked at 27 lb/bu, followed by high moisture corn and dry rolled corn. Meanwhile, starch degraded at 2-hour was numerically higher for high moisture corn, followed by corn steam-flaked at 27 lb/bu and dry-rolled corn. Gas production curves may depict a more complete picture of ruminal starch availability, where high moisture corn had the highest cumulative gas production after 24 hours, followed by corn steam-flaked at 27 lb/bu, and dry rolled corn.

Grain processing such as steam-flaking can improve grain digestibility (Orskov et al., 1969; McKnight et al., 1973; Galyean et al., 1976; Lee et al., 1982; Stock et al., 1987). In this study, high moisture corn presented higher *in vitro* gas production after 24 hours and rate of degradation than dry rolled corn. These results are in agreement with Stock et al. (1987), where high moisture corn was rapidly degraded in the rumen, while dry rolled corn was slowly degraded. Galyean et al. (1976) demonstrated the effect of grain processing when *in vitro* gas production and *in vivo* starch digestibility were higher for steam-flaked corn, followed by high-moisture corn and dry rolled corn.

The α-amylase enzyme acts on starch substrates in a multi-attack mechanism, where the enzymes bind to the substrate forming a complex to generate the first cleavage. After the first cleavage, the enzyme remains with the fragment of the original starch structure and catalyzes the hydrolysis of additional bonds yielding oligosaccharides before its dissociation (Robyt and French, 1967). The rate which amylase acts on starch substrates can vary depending on starch source, grain processing, granule size, amylose/amylopectin ratio, degree of crystallization, and other factors (Kayisu and Hood, 1979; Snow and O'Dea, 1981; Dreher et al., 1984; Hoover and Sosulski, 1985; Wursch et al., 1986; Ring et al., 1987; Ring et al., 1988). By grinding all substrates to 1 mm, we controlled the effect of particle size in this study. Our study demonstrated that the inclusion of Amaize can significantly increase the rate of degradation of different types of corn. In this study, the rate of degradation significantly increased when dry rolled corn was incubated with Amaize. Meanwhile, these parameters decreased when corn steam-flaked at 27 lb/bu was the substrate incubated with Amaize. These results were not expected since α-amylase has a lower hydrolytic capacity on crystalline starch than gelatinized starch (Hoover and Sosulski, 1985; Ring et al., 1987; Ring et al., 1988; Zinn et al., 2002). Processed grains typically have lower crystalline starch content and higher gelatinized starch content than dry rolled grains due to disruption of the crystalline starch granules during its heat processing (Baldwin et al., 1994). These differences in starch structure and availability might not be the factors driving the effects of Amaize on different types of processed corn.

Starch availability measures the difference between enzymatically-available starch and the total starch content of feedstuffs, where it can determine the degree of starch gelatinization and potentially estimate ruminal starch digestion (Schwandt et al., 2016;

Trotta et al., 2021a, 2021b). In this study, starch availability decreased as flake density increased and was lower for retrograded steam-flaked corn than non-retrograded steam-flaked corn, as expected. Schwandt et al. (2016) obtained similar results to our study, where starch availability of steam-flaked corn linearly decreased as flake density increased, ranging from 65% (24 lb/bu) to 37% (31 lb/bu). In agreement with the results of this study, Trotta et al. (2021a) and Trotta et al. (2021b) observed that enzymatic starch availability of steam-flaked corn decreases with retrogradation and increasing flake densities.

Increasing flake density of steam-flaked corn generally decreases ruminal and total tract starch digestibility (Theurer et al., 1999; Zinn et al., 2002; Ponce et al., 2013). In our study gas production and percent starch degraded at 2-hour of incubation were numerically higher for corn steam-flaked at 23 lb/bu, followed by corn steam-flaked at 25 lb/bu, corn steam-flaked at 29 lb/bu, corn steam-flaked at 31 lb/bu, and corn steam-flaked at 27 lb/bu. Meanwhile, available starch was numerically higher for corn steam-flaked at 23 lb/bu, followed by corn steam-flaked at 25 lb/bu, corn steam-flaked at 29 lb/bu, corn steam-flaked at 27 lb/bu, and corn steam-flaked at 31 lb/bu. Our data demonstrate that available starch did not accurately describe starch degradation across the different feedstuffs and steam-flaked corn with varying flake densities, suggesting that *in vitro* gas production might be a better tool to estimate ruminal starch digestion than enzymatic starch availability. *In vitro* gas production after 24-hour incubation and starch degraded after 2-hour incubation decreased as flake density increased except for density 27 lb/bu, in which these parameters were lower than expected. These results could be due to the lower starch availability this flake density presented since starch availability correlates to the rate of ruminal starch fermentation (Schwandt et al., 2016).

In our study, retrograded steam-flaked corn had a lower rate of degradation than non-retrograded steam-flaked corn, but conversely, it had higher *in vitro* gas production after 24-hour incubation. Our data suggests that ruminal digestibility of steam-flaked corn may not be constrained by starch retrogradation but rather, changes in rate and passage may be the primary constraints. Our data also indicate that starch retrogradation reduces the ruminal rate of degradation, possibly increasing the amount of starch that escapes the rumen and enters the small intestine (Theurer, 1986; Theurer et al., 1999; Owens et al., 2016; Trotta et al., 2021b). Retrogradation creates rigidity and crystallization of starch which can reduce the amylase rate of hydrolysis (Miles et al., 1985; Zhang and Jackson, 1992; Biliaderis, 1998). The reduction in amylase hydrolysis rate could be the reason why, in our study, we observed lower starch degraded and *in vitro* gas production after the 2-hour incubation in retrograded steam-flaked corn when compared to non-retrograded steam-flaked corn.

When analyzing the effects of Amaize in retrograded and non-retrograded steam-flaked corn, we observed that the rate of degradation and *in vitro* gas production after 2-hour incubation increased when retrograde steam-flaked corn was incubated with Amaize. It is possible that Amaize did not improve rate of degradation for non-retrograde steam-flaked corn due to its already high starch availability content and greater susceptibility to microbial enzymatic attack (Rooney and Pflugfelder, 1986; Zinn et al., 2002). The effect of α-amylase in ruminal starch degradation can vary. Noziere et al. (2013) reported improvements in ruminal starch digestion when supplementing first-lactating cows with exogenous amylase. Rojo et al. (2005) also observed an increase in ruminal starch digestion when supplementing amylase from direct-fed microbial to lambs. Tricarico et al. (2008)

did not detect improvements in ruminal starch digestion when comparing studies that supplemented *Aspergillus oryzae* extract containing α-amylase activity to ruminants *in vivo* and *in vitro*. In our study, Amaize increased the percent starch degraded after 2-hour incubation for both retrograded and non-retrograded steam-flaked corn. Our data imply that Amaize could be used to increase the amount of starch degraded in the rumen, possibly reducing the starch flow to the small intestine when feeding retrograded or non-retrograded steam-flaked corn to cattle.

In summary, *in vitro* gas production appears to more accurately estimate ruminal starch degradation of different feedstuffs than starch availability. Grain processing improves *in vitro* gas production and starch degradation parameters of multiple feedstuffs. Amaize can potentially increase ruminal *in vitro* rate of degradation and starch degradation of different feedstuffs. Corn steam-flaked at lower flake densities present higher ruminal *in vitro* gas production and starch degradation. Ruminal digestibility of steam-flaked corn after 24-hour incubation is not limited by starch retrogradation. Starch retrogradation reduces the ruminal rate of degradation, possibly increasing the amount of starch that escapes the rumen and enters the small intestine.

4.5. Conclusion

Starch availability increases with steam-flaking of grains, and decreases with retrogradation and increasing flake density. *In vitro* gas production after 24-hour incubation increases when Amaize is incubated with dry rolled barley but decreases when the substrates are steam-flaked barley and retrograded steam-flaked corn. Amaize

supplementation does not cause any changes in *in vitro* gas production after 24-hour incubation when the substrates are dry rolled corn, high moisture corn, and steam-flaked corn. Amaize increases *in vitro* rate of degradation when incubated with corn steam-flaked at 26 lb/bu, steam-flaked barley, dry rolled corn, and retrograde steam-flaked corn. But Amaize decreases this parameter when incubated with corn steam-flaked at 27 lb/bu. *In vitro* percent starch degraded increased when Amaize was incubated with steam-flaked corn and retrograded steam-flaked corn, but no changes occurred when incubated with dry rolled corn, high moisture corn, dry rolled barley, and steam-flaked barley. Our study suggests that *in vitro* gas production might be a more descriptive tool to estimate starch degradation in the rumen than enzymatic starch availability measurements. Our data also indicate that Amaize can increase the *in vitro* rate of degradation and starch degradation of different substrates with different grain processing methods, potentially increasing starch degraded in the rumen and lowering starch flow to the small intestine.

Table 4.1. Composition of diet fed *ad libitum* to rumen fluid donor animals in *in vitro* study.

Feedstuff	% DM	Nutrient Concentration, % DM	
Rye Silage	57.00	TDN, %	74.50
Dry Rolled Corn	35.03	NEm, Mcal/kg	1.76
Soybean Meal	6.76	MP, %	10.50
Calcium Carbonate	0.69	CP, %	13.90
Premix[1]	0.41	DIP, % of CP	27.00
Tallow	0.09	UIP, % of CP	73.00
Rumensin-90	0.02	Ca, %	0.47
Total	100.00	P, %	0.33
		Ca:P	1.42

[1]Salt (92.86 PCT), Sulfur (1.20 PCT), Calcium (0.06 PCT), Iron (9,290.2 ppm), Zinc (5,520.2 ppm), Manganese (4,792.3 ppm), Copper (1,837.7 ppm), Iodine (119.9 ppm), Cobalt (68.9 ppm) and Selenium (18.5 ppm)

Table 4.2. Starch content and availability on a dry-matter basis (%) of substrates used for *in vitro* gas production analysis and starch degradation (Experiment 1, 2 and 3).

	Feedstuff	Total Starch, %	SEM[1]	Starch Availability, %	SEM[1]
Experiment 1	Steam-flaked corn 26 lb/bu	74.80	0.42	51.49	0.30
	Steam-flaked corn 32 lb/bu	75.01	0.66	44.99	0.57
	Dry Rolled Barley	58.39	1.28	7.36	0.21
	Steam Flaked Barley	58.81	0.46	22.98	0.55
Experiment 2	Dry Rolled Corn	74.91	0.60	9.47	0.11
	High Moisture Corn	74.81	0.22	13.54	0.35
	Steam-flaked corn 27 lb/bu	74.96	0.16	48.16	0.47
Experiment 3	Steam-flaked corn 23 lb/bu	74.76	0.70	54.71	0.39
	Retrograde steam-flaked corn 23 lb/bu	74.55	0.29	42.01	0.49
	Steam-flaked corn 25 lb/bu	74.44	0.22	52.89	0.40
	Retrograde steam-flaked corn 25 lb/bu	74.89	0.26	39.55	0.09
	Steam-flaked corn 27 lb/bu	74.96	0.16	48.16	0.47
	Retrograde steam-flaked corn 27 lb/bu	75.07	0.45	38.84	0.43
	Steam-flaked corn 29 lb/bu	74.68	0.17	49.55	0.62
	Retrograde steam-flaked corn 29 lb/bu	74.98	0.06	37.50	0.23
	Steam-flaked corn 31 lb/bu	74.45	0.26	46.10	0.97
	Retrograde steam-flaked corn 31 lb/bu	74.72	0.15	36.89	0.23

[1]SEM: standard error of the mean.

Table 4.3. Effects of substrate and α-amylase (Amaize) inclusion on *in vitro* gas production parameters and starch degradation between corn steam-flaked at 26 and 32 lb/bushel, dry rolled barley and steam-flaked barley (Experiment 1).

Substrate	Steam-flaked corn 26		Steam-flaked corn 32		Dry rolled barley		Steam-flaked barley			P-value		
Amaize Inclusion, IU/100 mL	0 IU	15 IU	0 IU	15 IU	0 IU	15 IU	0 IU	15 IU	SEM[1]	Substrate	Amaize	Substrate x Amaize
Gas Production – 2 h, mL	37	38	39	41	32	32	40	47	4	0.2890	0.2758	0.6399
Starch Degraded – 2 h, %	30.34^{e}	30.78^{d}	32.05^{c}	33.21^{b}	28.99^{f}	29.26^{f}	34.11^{a}	34.46^{a}	0.09	< 0.001	< 0.001	0.0002
Gas Production – 24 h, mL	482^{b}	501^{b}	503^{b}	495^{b}	440^{c}	477^{b}	590^{a}	562^{b}	5	< 0.001	0.2333	< 0.001
Rate of Degradation – 24 h, %/h	15.18^{c}	15.94^{b}	14.50^{d}	14.38^{d}	18.94^{a}	18.74^{a}	9.16^{f}	10.57^{e}	0.05	< 0.001	< 0.001	< 0.001

Amaize Inclusion: IU/100 mL inoculum.

[1]SEM: standard error of the mean, n = 12.

Effect of other interactions were not significant ($P > 0.05$).

[abcdef]Least squares means that do not contain at least one of the same letters across rows and columns differ ($P < 0.05$).

Table 4.4. Effects of corn type and α-amylase (Amaize) inclusion on *in vitro* gas production parameters and starch degradation between dry rolled corn, high moisture corn and steam-flaked at 27 lb/bushel (Experiment 2).

Corn Type	Dry rolled corn		High moisture corn		Steam-flaked corn 27		SEM[1]	P-value		
Amaize Inclusion, IU/100 mL	0	15	0	15	0	15		Corn type	Amaize	Corn type x Amaize
Gas Production – 2 h, mL	35	33	37	33	31	38	3	0.8454	0.9000	0.1568
Starch Degraded – 2 h, %	34.74^{b}	34.27^{b}	35.79^{a}	36.32^{a}	34.86^{ab}	36.42^{ab}	0.48	0.0123	0.1894	0.1373
Gas Production – 24 h, mL	450^{d}	458^{dc}	524^{a}	514^{a}	493^{b}	474^{bc}	4	< 0.001	0.0920	0.0297
Rate of Degradation – 24 h, %/h	13.08^{e}	13.96^{d}	15.32^{b}	15.49^{ab}	15.54^{a}	14.74^{c}	0.05	< 0.001	0.0370	< 0.001

[1]SEM: standard error of the mean, n = 10.

[abcde]Least squares means that do not contain at least one of the same letters across rows differ (P < 0.05).

Table 4.5. Effects of flake density on *in vitro* gas production parameters and starch degradation between steam-flaked corn and retrograde steam-flaked corn at 23, 25, 27, 29, and 31 lb/bushel (Experiment 3).

Flake Density, lb/bushel	23	25	27	29	31	SEM[1]	P-value			
							Linear	Quadratic	Cubic	Quartic
Gas Production – 2 h, mL	44[a]	42[ab]	35[c]	39[bc]	38[c]	1	< 0.001	0.0027	0.8655	0.0007
Starch Degraded – 2 h, %	35.25[a]	31.90[b]	25.09[d]	28.13[bc]	26.21[c]	0.40	< 0.001	< 0.001	0.3428	0.3319
Gas Production – 24 h, mL	525[a]	501[b]	488[c]	505[b]	510[b]	3	0.0045	< 0.001	0.0096	0.0109
Rate of Degradation – 24 h, %/h	13.70[d]	14.17[b]	14.32[a]	13.87[c]	13.42[e]	0.03	< 0.001	< 0.001	0.0012	0.0004

[1]SEM: standard error of the mean, n = 24.

[abcde]Least squares means that do not contain at least one of the same letters across rows and differ (P < 0.05).

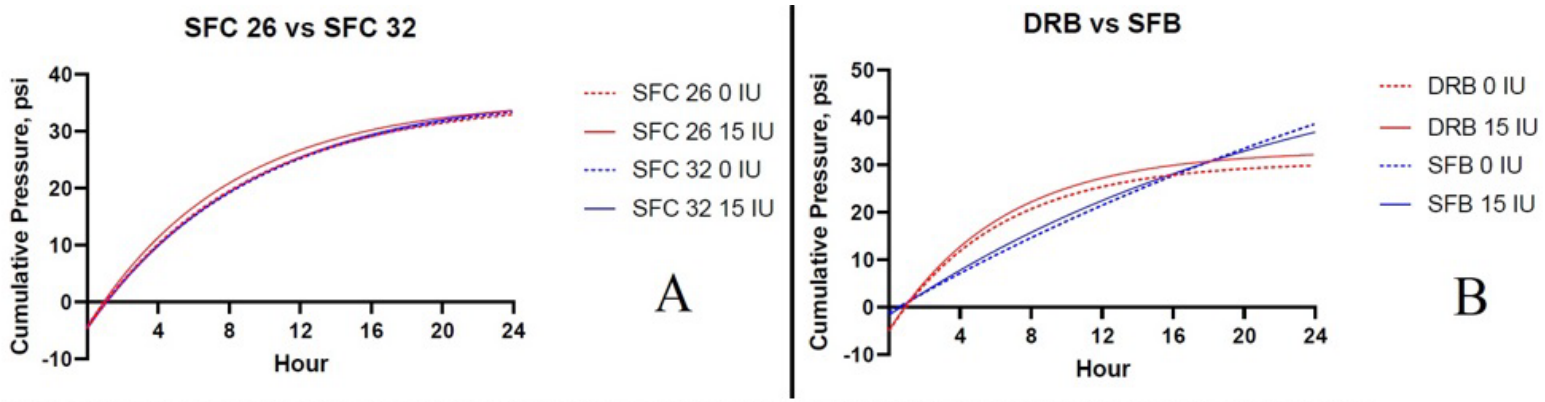
Table 4.6. Effects of retrogradation and α-amylase (Amaize) inclusion on *in vitro* gas production and starch degradation between steam-flaked corn and retrograde steam-flaked corn (Experiment 3).

Retrogradation	Steam-flaked		Retrograde		SEM[1]	P-value		
Amaize Inclusion, IU/100 mL	0 IU	15 IU	0 IU	15 IU		Retrogradation	Amaize	Retrogradation x Amaize
Gas Production – 2 h, mL	41[a]	42[a]	36[b]	38[a]	1	0.0055	0.0134	0.0383
Starch Degraded – 2 h, %	34.98[b]	39.10[a]	20.42[d]	22.75[c]	0.36	< 0.001	< 0.001	0.0138
Gas Production – 24 h, mL	488[c]	492[c]	529[a]	514[b]	3	< 0.001	0.0544	0.0003
Rate of Degradation – 24 h, %/h	15.50[a]	15.55[a]	11.76[c]	12.77[b]	0.03	< 0.001	< 0.001	< 0.001

[1]SEM: standard error of the mean, n = 24.

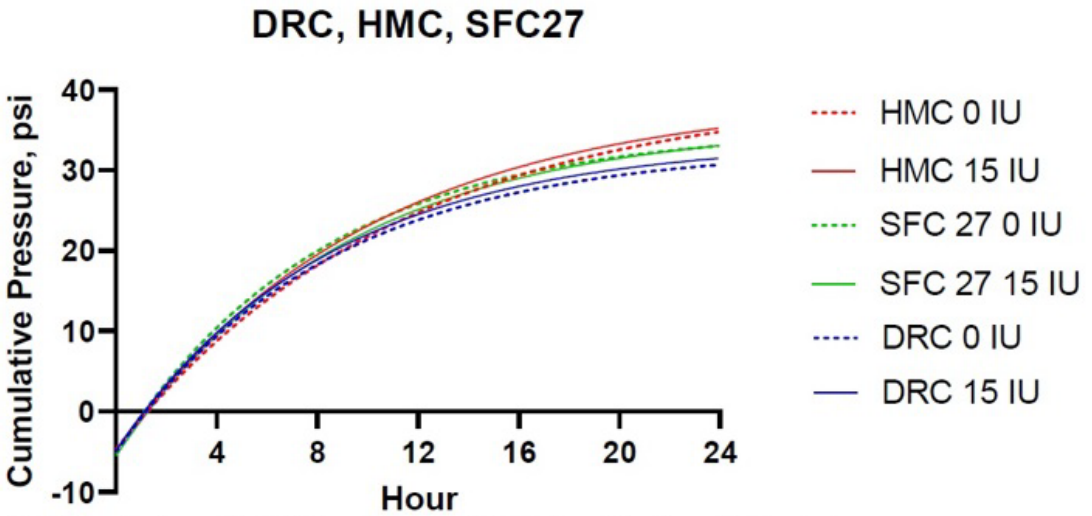
Effect of other interactions were not significant ($P > 0.05$).

[abc]Least squares means that do not contain at least one of the same letters across rows and differ ($P < 0.05$).



SFC 26: Steam-flaked corn 26 lb/bushel; SFC 32: Steam-flaked corn 32 lb/bushel; DRB: Dry rolled barley; SFB: Steam-flaked barley.
0 IU: Amaize 0 IU/100 mL inoculum; 15 IU: Amaize 15 IU/100 mL inoculum.

Figure 4.1. *In vitro* gas production curve of corn steam-flaked at 26, corn steam-flaked at 32 lb/bushel, dry rolled barley, and steam-flaked barley after 24-hour incubation (Experiment 1).



DRC: Dry rolled corn; HMC: high moisture corn; SFC27: Steam-flaked corn 27 lb/bushel.
0 IU: Amaize 0 IU/100 mL inoculum; 15 IU: Amaize 15 IU/100 mL inoculum.

Figure 4.2. *In vitro* gas production curve of dry rolled corn, high moisture corn, and corn steam-flaked at 27 lb/bushel after 24-hour incubation (Experiment 2).

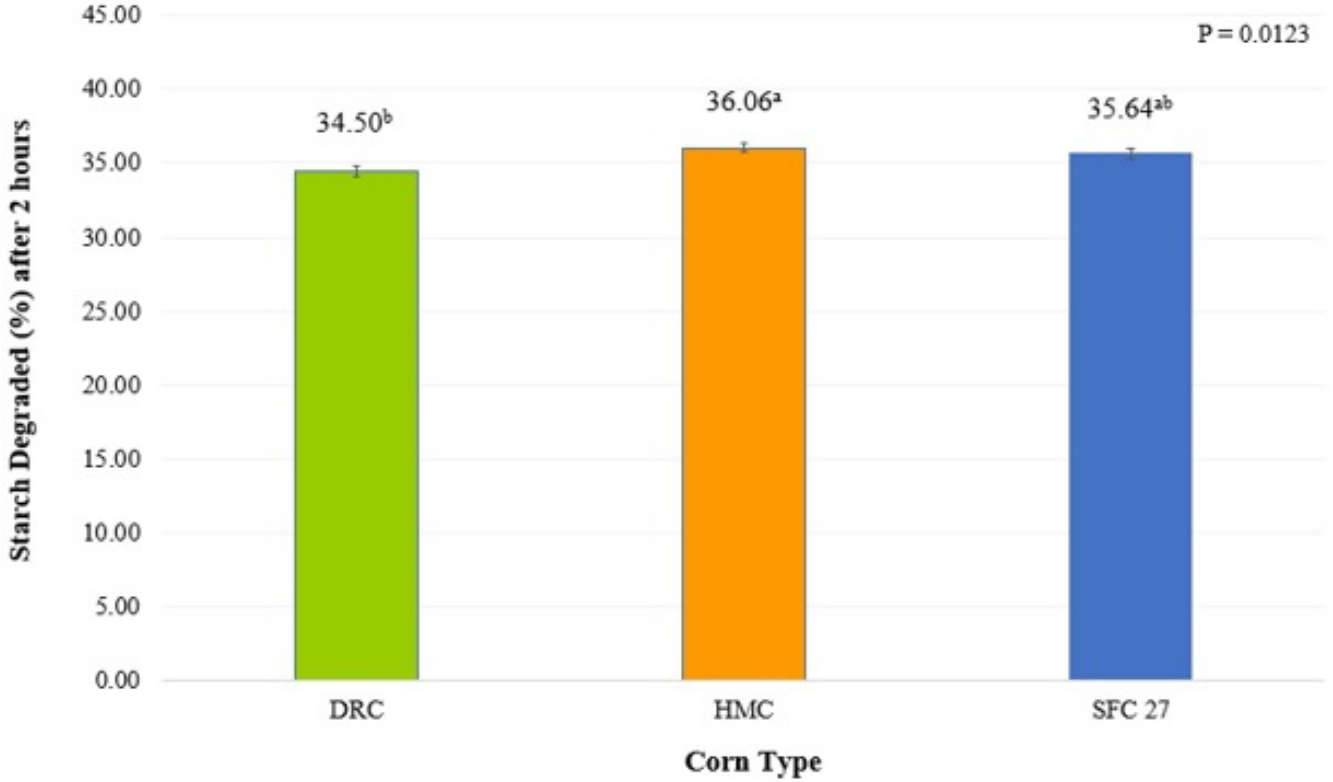
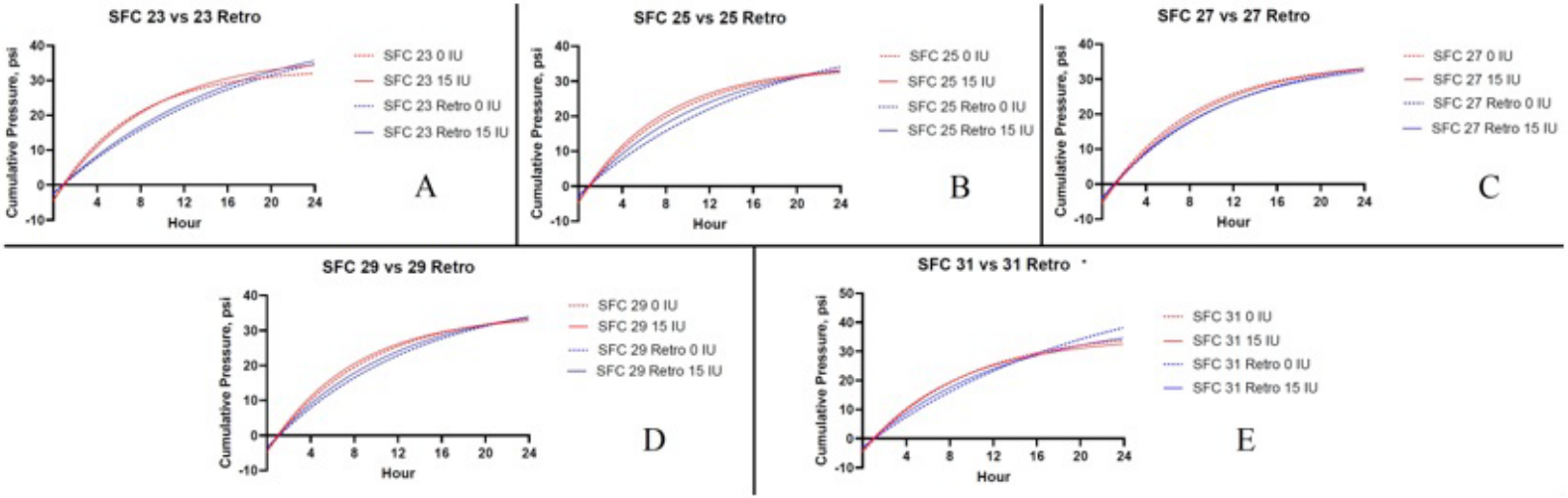


Figure 4.3. Effects of corn type on in vitro starch degradation after 2-hour incubation between dry rolled corn, high moisture corn, and corn steam-flaked at 27 lb/bushel (Experiment 2).



DRC: Dry rolled corn; HMC: High moisture corn; SFC 23: Steam-flaked corn 23 lb/bushel; 23 Retro: Retrograde steam-flaked corn 23 lb/bushel; SFC 25: Steam-flaked corn 25 lb/bushel; 25 Retro: Retrograde steam-flaked corn 25 lb/bushel; SFC 27: Steam-flaked corn 27 lb/bushel; 27 Retro: Retrograde steam-flaked corn 27 lb/bushel; SFC 29: Steam-flaked corn 29 lb/bushel; 29 Retro: Retrograde steam-flaked corn 29 lb/bushel; SFC 31: Steam-flaked corn 31 lb/bushel; 31 Retro: Retrograde steam-flaked corn 31 lb/bushel.
0 IU: Amaize 0 IU/100 mL inoculum; 15 IU: Amaize 15 IU/100 mL inoculum.

Figure 4.4. *In vitro* gas production curve of steam-flaked corn and retrograde steam-flaked corn at 23, 25, 27, 29, and 31 lb/bushel after 24-hour incubation (Experiment 3).

CHAPTER 5.

Summary and Conclusions

Metabolic disorders can occur in ruminants during the transition period from a high-forage diet to a high-grain diet. Finishing diets typically used in feedlots are high in concentrate which can increase the risk for rumen acidosis and liver abscess. Dietary antibiotics such as monensin and tylosin can be used to prevent these physiological complications. Due to growing concerns over the use of antibiotics in agriculture, alternative products have been developed with the goal to mitigate digestive distress that might occur when cattle consume a high-concentrate diet. This dissertation aimed to understand the physiological mechanisms of Programmed Nutrition Beef Finisher and Amaize (α-amylase) supplementation in the rumen and whole-body of cattle.

To accomplish these goals, measurements of animal metabolism and performance were conducted *in vivo*, and changes in rumen metabolism were determined *in vitro*. The first portion of this dissertation compared the effects of Programmed Nutrition Beef Finisher versus traditional feedlot additives monensin and tylosin *in vivo*. The study demonstrated that dry matter digestibility and average daily gain does not improve with the supplementation of monensin and tylosin, Programmed Nutrition Beef Finisher, or their combination. These results indicate that Programmed Nutrition Beef Finisher is not recommended to enhance animal performance.

Monensin and tylosin can improve acid detergent fiber (ADF) digestibility, meanwhile supplementing steers with a combination of Programmed Nutrition Beef

Finisher, monensin and tylosin can lead to a decrease in acid detergent fiber digestibility. It is important to emphasize that in this study animals consumed a high-grain diet, where different responses could be obtained if animals were consuming a high-forage diet due to potential microbial population adaptation towards a more fibrolytic rumen environment. It was also shown that feeding the combination of these additives can increase the time rumen pH remains below 5.6 and 5.3, which can lead to the negative effects of rumen acidosis. When rumen pH remains below 5.3 for a prolonged period of time it increases the risk of occurring rumen wall damage.

Programmed Nutrition Beef Finisher would not be recommended to reduce methane emissions in animals consuming a finishing feedlot diet, since no differences in methane production were detected among treatments. The control treatment presented greater retained energy and energy retained in fat than monensin and tylosin, Programmed Nutrition Beef Finisher, and their combination. Our results indicate that these additives do not increase energy deposited as fat in cattle consuming a high-grain diet when compared to a conventional trace mineral supplement. Similarly, no differences in energy retained in protein, protein turnover, or protein synthesis were detected among the treatments. Our data demonstrates that Programmed Nutrition Beef Finisher would not improve muscle synthesis and deposition when supplemented to cattle consuming a diet composed primarily of high moisture corn and corn silage.

The second portion of this dissertation analyzed the effects of Amaize (α-amylase) supplementation, grain processing, grain flake density, and grain retrogradation on rumen metabolism and starch degradation *in vitro*. Our data demonstrate that *in vitro* gas production better predicted starch degradation in the rumen than enzymatic starch

availability measurements, indicating that *in vitro* gas production might be a more accurate method to predict ruminal starch degradation.

In our study, Amaize improved *in vitro* gas production after 24-hour incubation for dry rolled barley but caused a reduction in gas production for steam-flaked barley and retrograded steam-flaked corn. This indicates that Amaize could potentially increase ruminal degradation of dry rolled grains that are slowly colonized and digested in the rumen. Amaize increases *in vitro* rate of degradation of corn steam-flaked at 26 lb/bu, steam-flaked barley, dry rolled corn, and retrograde steam-flaked corn. Our results establish that Amaize can be used to increase the ruminal rate of degradation across a range of grain types and processing methods. Amaize could be used to improve the ruminal rate of degradation when animals are fed grains that are processed with heat (steam-flaking) and no heat (dry rolling).

Our data indicate that Amaize can be used to increase *in vitro* percent starch degraded in both steam-flaked corn and retrograded steam-flaked corn. These results imply that the degree of starch crystallization and gelatinization might not limit the effectiveness of Amaize on these parameters. Overall, our data suggest that Amaize can potentially be used to increase ruminal substrate and starch degradation across a range of grain types with different processing methods.

In conclusion, Programmed Nutrition does not appear to be an adequate replacement for monensin and tylosin to alleviate the negative effects of a high-grain diet in the rumen or to improve animal performance. Amaize could be used to improve ruminal substrate digestibility over 24 hours, increase ruminal substrate rate of degradation, and percent starch degraded in the rumen across multiple grain types submitted to different

processing methods. It is important to emphasize that these results are limited to cattle consuming high-grain diets with limited adaptation periods to treatments. By better understanding the mechanisms of these supplements across a range of diets and substrates we can optimize their response in animal health and performance.

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VITA

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