Effect of exogeneous enzymes on performance of finishing cattle fed sorghum-based diets

by

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Abstract

Interest in water conservation efforts has resulted in increased interest in grain sorghum. One limitation to sorghum utilization is it decreased digestibility compared to corn. Mitigating this challenge was the motivation for the following evaluations. A finishing study examining Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, KS) paired with an *in vitro* experiment examining four differing protease enzymes. The finishing experiment utilized 280 crossbred steers (441.74 kg ± 15.19 kg initial body weight) to evaluate nutrient digestibility, feedlot performance, and carcass characteristics. Steers were randomly allocated to 36 feedlot pens containing 7 (8 pens) or 8 (28 pens) animals per pen. Pens were randomly assigned to one of two dietary treatments within blocks (18 blocks). Cattle were randomly assigned to a pen within each block by chute order, two pens were filled at a time. The finishing diet consisted of 59.62% ground sorghum, 25% sorghum-based dried distillers grains with solubles, 12% corn silage, 3.38% supplement. Ground corn was utilized as the carrier for the supplement; within the enzyme groups supplement Pyramid PowerGrow Ruminant was used in lieu of the ground corn. Cattle were fed once daily in the morning, *ad libitum*, and daily feed bunk scores were taken to determine feed allocation. On day 67, fresh fecal pats were collected from the pen surface and analyzed for protein, starch, neutral detergent fiber (NDF), ash, organic matter, and acid detergent insoluble ash concentrations. After 148 days on feed, steers were harvested, and after 96 h of refrigeration carcass data were collected. Data were analyzed as mixed models with diet as the fixed effect, pen as the experimental unit, and block as the random effect. There were numerical but non-significant ($P > 0.10$) differences between treatments for feedlot performance, nutrient digestibility, and carcass characteristics. Final body weight of the enzyme treatment was 5.4 kg heavier than the control treatment. Starch digestibility was marginally higher but not

significantly different in the enzyme group compared to the control group (91.26% vs. 89.58%). Marbling score was 15.8 points higher in the control treatment compared to the enzyme treatment. The *in vitro* experiment evaluated effects of four protease enzymes on *in vitro* dry matter digestibility (IVDMD), terminal pH, volatile fatty acid (VFA) production, and methane production per gram of substrate digested. Liquipanol (liquid papain derived from *carica papaya* L.), fungal protease, fungal neutral protease, and fungal acid proteases were examined when added to sorghum at a rate of 3 or 30 μL per tube. There were 11 treatments, 9 of which utilized sorghum as a substrate, those treatments were fungal acid protease 3 and 30 μL per 50-mL tube, fungal protease 3 and 30 μL per 50-mL tube, fungal neutral protease 3 and 30 μL per 50-mL per tube, Liquipanol 3 and 30 μL per 50-mL tube, and sorghum no enzyme. There was also a corn no enzyme and a blank treatment. The fungal acid protease, fungal protease, fungal neutral protease, and Liquipanol treatments all utilized sorghum as the substrate. A randomized complete block design was utilized to evaluate the 11 treatments including a control corn and sorghum treatment and nine blocks. Terminal pH, IVDMD, and VFA profiles were analyzed as mixed models with treatment as the fixed effect and run and block within run as random effects. The control corn treatment had the lowest pH, and greatest IVDMD, and greatest VFA production compared to the sorghum-based treatment groups ($P < 0.01$). The results of these two experiments indicate that protease enzyme supplementation results in modest but statistically insignificant differences in sorghum digestibility.

Key Words: Feedlot, Sorghum, Protease Enzymes, *In Vitro*

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Dedication

I want to dedicate my thesis to my family, who has always believed in me and supported my dreams no matter how large or potentially unattainable. Your encouragement means the world to me.

Chapter 1 - Literature Review - Sorghum starch chemistry and impacts of processing and enzyme supplementation

E. R. Kiselewski and J. S. Drouillard

Abstract

A majority of beef cattle within the United Stares are produced throughout the Great Plains region. Within this region lies the Ogallala Aquifer, which provides the US with 30% of the irrigated water. Due to irrigation practices, water within the aquifer is being depleted quicker than able to be recharged. With the increased need for water conservation, there is a need for alternative crops that require less water. One option is to plant grain sorghum which can be produced with less water. One limitation to sorghum is that it is less digestible than corn due to the starch-protein matrix. This matrix limits the ability of amylolytic enzymes from being able to break down starch [1]. Traditionally, dry-rolled grain sorghum has only 92% NE_m of corn [2]. Through different processing methods, the digestibility of sorghum is able to be increased to be more comparable with steam-flaked corn. Chen et al. (1994) [3] examined steam-flaked corn and sorghum for their comparative impact the performance of lactating dairy cows and determined that there was a 21% increase in NE_L for steam flaked sorghum compared to ground sorghum. Other than steam flaking sorghum, another potential way to increase sorghum starch digestibility is with addition of protease enzymes. Protease enzymes are able to hydrolyze peptide bonds, leading to the degradation of protein within a substrate [4]. The use of exogeneous enzymes have had varying responses [5]. Vera et al. (2012) [6] reported increased nutrient digestibility when steers were supplemented with exogeneous protease enzymes during the finishing phase. Feeding grain sorghum has some limitations, but with supplementation and (or) grain processing sorghum could be a beneficial feedstuff to use in place of or in combination with corn.

Key words:

Grain sorghum, Starch digestibility, Protease enzymes

1.1 Introduction

Carbohydrates are a very important part of any ruminant diet. Carbohydrates are able to provide an animal, as well as the microbes within the rumen, necessary digestible energy. This class of nutrients is also able to provide the rumen with the necessary stimulation for ruminal activity and motility. Ruminal stimulation occurs when pieces of fibrous material scratch the rumen wall. Carbohydrates are broken up broadly into non-neutral detergent fiber (non-NDF) and neutral detergent fiber (NDF). Within the non-NDF classification of carbohydrates is starch. Starch is an integral part of finishing cattle diets [7]. Cereal grains like corn and sorghum are high in starch. Compared to corn, sorghum is an underutilized cereal grain within livestock diets. Sorghum may be advantageous in some geographies due to its ability to grow during times of drought and lower water requirement compared to corn [8]. The starch-protein matrix within sorghum makes the grain difficult for ruminants to digest. To break up the starch-protein matrix a few of different processing methods that have been utilized are grinding/dry rolling, steam-flaking, and ensiling. Steam-flaking sorghum has had the most promise to increase starch digestibility to make the grain more comparable to steam-flaked corn [9]. Protease enzymes are able to hydrolyze peptide bonds, leading to the degradation of protein within a substrate [4]. The use of exogeneous enzymes have had varying responses, with not all studies reporting improvements [5].

1.2 Starch Structure and Biochemistry

A major source of energy in beef cattle diets is carbohydrates. Within plants the main storage carbohydrates is starch [7]. Starch is used for long term energy storage and is found in seeds, tubers, roots, fruits, leaves, as well as pollen [10].

1.2.2 Digestion of Starch

Starch degradation begins within the rumen. Small amounts are degraded within the small intestine, and the remainder is fermented within the hindgut [11]. In the rumen, a large portion of starch is broken down and fermented by ruminal bacteria, with a portion being broken down by protozoa and fungi [12]. Within the rumen, amylolytic bacteria are considered the main starch-degrading microorganisms followed by protozoa and fungi. *Streptococcus bovis, Bacteriodes amylophilus,* and *Selenomonas ruminantium* are some of the commonly found starch-utilizing bacteria within the rumen. *Streptococcus bovis* is able to digest heated starch quickly and is a lactic acid producer. *Bacteriodes amylophilus* breaks down starch as well as di- and oligosaccharides by attaching to the substrate. When present within the rumen *Bacteriodes amylophilus* can account for 10% of the total bacteria within the rumen and is a predominant starch digester. *Selenomonas ruminantium* ferments many types of sugars and can grow in a variety of rumen conditions. The main end products produced by amylolytic bacteria within the rumen are acetate, propionate, butyrate, and lactate[11,13]. Starch-fermenting bacteria produce greater proportions of propionic acid (35-45 mol/100 mol VFA) compared to cellulose or hemicellulose fermenting bacteria (15-20 mol/100 mol VFA). Propionic acid is a hydrogen sink product and is advantageous to produce due to the utilization of hydrogen within the rumen rather than being metabolized into methane [14]. Amylolytic protozoa degrade starch by engulfing starch molecules to produce H_2, CO_2, acetate, butyrate, and glycerol. Protozoa are able to slow down starch-fermentation due to the ability to engulf starch molecules which effectively sequesters starch to avoid rapid degradation by bacteria. Once those molecules are engulfed, protozoa need a maximum of 36 hours to metabolize starch. *Eremoplastron bovis, Diplastron affine, Ophryoscolex caudatus,* and *Polyplastron multiesiculatum* are protozoa with high

amylolytic activities [11]. Fungi play an important role in ruminal starch digestion by creating lesions in the surface of the feedstuff, allowing bacteria to attach to more areas of the feedstuff [12]. Within the small intestine starch is absorbed and digested within three steps. Step one begins in the duodenum. Pancreatic α-amylase initiates starch breakdown by producing maltose and branched-chain products. Step two occurs at the brush border membrane by brush border carbohydrase. The carbohydrases further break down the branched-chain products into glucose to be able to be metabolized onto VFA and utilized by the animal for energy. During the third phase of intestinal starch digestion, the glucose is transported out of the lumen into the portal circulation [15]. Starch that is not digested within the small intestine passes to the large intestine where it can potentially be fermented to produce VFA or lactate as end products [14].

1.2.3 Bacterial Breakdown of Starch

Ruminal bacteria cannot ingest starch molecules but produce enzymes that are able to break down amylose and amylopectin. There are three main types of enzymes produced by amylolytic bacteria: endoamylases, exoamylases, and debranching enzymes. Endoamylases are able to cleave α-1,4 glycosidic linkages randomly in the interior region of the starch polymer or oligosaccharides [11]. α -Amylase is found in a widespread number of ruminal bacteria. α -Amylase hydrolyzes α-1,4- linkages. The optimum pH for α -amylases is 2.0 to 10.5. α-1,6-glucosidic linkages of glycogen, amylopectin, and oligosaccharides are hydrolyzed by isoamylase. Pullulan cannot be degraded by isoamylases. Pullulanases are debranching enzymes that hydrolyze 1,6-linkages of pullulan and oligosaccharides [16]. Glucoamylases are able to form glucose as an end product by degrading both 1,4- and 1,6-linkages [11]. Post ruminal digestion of starch includes pancreatic α -amylase. Pancreatic α -amylase is released in response to dietary carbohydrates such as starch, sucrose, fructose, and glucose [17].

1.2.4 Limitations to Starch Digestion

Starch digestion can be limited by the protein-starch interactions, physical form of the starch, and antinutritional components within the feed [18]. Starch retrogradation can be inhibited or promoted based on the exposed protein residues. Starch retrogradation can be promoted by the formation of disulfide linkages (covalent bonds) between proteins. The formation of glycosidic bonds during high-temperature processing can inhibit starch retrogradation [19]. Amylose content within the starch granule is inversely related to the digestibility of starch [20]. A study by [21] examined the *in vitro* digestibility of corn starches with varying amylose content. The study examined waxy corn starch (amylose content 3.32%), corn starch (amylose content 20.13%), high amylose corn starch 50 (amylose content 51.22%), high amylose corn starch 60 (amylose content 61.26%), high amylose corn starch 70 (amylose content 71.16%), and high amylose corn starch 80 (amylose content 80.71%). The authors of this study reported that there was a strong instance of starch digestion resistance within the high amylose corn starches. When starches are digested too quickly, pH within the rumen can decrease quickly. Acute acidosis is a condition when the ruminal pH drops below 5.0. During acute acidosis there is an increase in the concentration of lactic acid of 50 to 120 mM. With the increase in lactic acid, there is an increase in lactic acid-producers and a decrease in lactic acid-utilizers [22]. Acidosis can not only result in changes in bacterial populations, but also can predispose animals to development of liver abscesses, ruminitis, polioencephalomalacia, and laminitis [23].

1.3 Limitations to Sorghum Digestion

Within sorghum grain, a limitation to starch digestion is the starch-protein matrix. Sorghum proteins are divided into two groups, kafirins and non-kafirins. Non-kafirin form around the protein bodies, forming a matrix around starch granules within the endosperm of the sorghum

kernel. The hydrophobic nature of kafirin proteins makes the starch-protein matrix resistant to enzymatic degradation. The nature of the matrix is able to physically limit amylase from accessing the starch granules within the matrix [1,24].

1.4 Use of Sorghum in Ruminant Diets

Sorghum grain ranks fifth in the world in grain production behind corn, rice, wheat and barley [25]. Sorghum grain is thought to be the second most common grain source used in feedlot diets. Herrera-Aldana et al. (1990) [26] incubated cereal grains for 48 hours to determine degradability. After the 48 hours, starch that was ruminally available was 49% for sorghum, and the ruminally available starch percent of corn was 62. The metabolizable energy (ME) value for corn is 3.42 while the ME value for sorghum is 3.22 [27].

1.4.1 Environmental Considerations for Sorghum

Within the High Plains region, water conservation efforts have become increasingly prominent due to water levels within the Ogallala Aquifer decreasing faster than able to be recharged. In areas where rainfall is limited, irrigation is a common practice. Sorghum grain is an option that is able to be grown in areas with limited rainfall. Sorghum grain requires 533 mm of water throughout the growing season compared to the 635mm of water corn requires to grow [8]. The grain is well adapted to grow in semi-arid regions of the world due to the plants' drought tolerance, and is able to grow well due to lower water requirement compared to other summer cereal grains [28]. The primary sectors sorghum grain is used in are livestock production, ethanol production, food products, pet food, and exports [29].

1.4.1.A Methane Production

Cattle produce more methane when the animal is ruminating compared to times when the animal is consuming grain or grass. Ruminant microbes produce methane by methanogenesis. This

process is done by archaea within the rumen. During the methanogenesis process, H_2 produced through other fermentative processes is utilized. When H_2 is diverted away from methanogenesis less methane is able to be produced within the rumen. Diverting H_2 away from methanogenesis is an advantageous process within the rumen, as enteric methane production constitutes a loss of energeticy. This loss of energy can represent up to 12% of gross energy intake [30]. [31]Animals on pasture were determined to produce 0.23 kg of CH_4 animal^{-1}day^{-1}, while animals on a high-concentrate diet produced 0.07 kg of CH_4 animal^{-1}day^{-1}. When cattle are on high-grain diets there is less methane produced compared to cattle on high-roughage diets. Within high-grain diets there are still differences among different grain types as well as processing methods for methane production. Cattle on a steam-flaked diet had a 20% reduction in methane production compared to cattle on a dry-rolled grain diet [7]. Processing is not the only dietary factor that can impact methane production. Time of year, amount of fats and oils in the diet, use of ionophores, as well as defaunation can decrease methane production. Adding fats and oils, feeding ionophores, and defaunation all decrease methane production by diverting H_2 away from methanogenesis through various methods [32].

1.4.2 Sorghum Processing

Grain processing is more important in cattle diets than sheep and goat diets. Cattle are less able to utilize whole grains than other ruminants [33]. Whole sorghum is less digestible compared to corn, wheat, and barley. Differing processing methods like mechanical processing, wet processing, heat and moisture processing, as well as hot, dry methods have been used to process sorghum grain. Processing sorghum is extremely beneficial for livestock diets. Whole grains are not very digestible within the rumen; bacteria are not able to attach to highly digestible regions of the grain. When grains are finely ground, the grain is able to be more rapidly digested. Feed

particles left unground are passed thorough the rumen quicker than particles that are able to be broken down and fermented. Processed grain is able to increase surface area, allowing more bacteria to attach to the grain. When processing the grain, the cell structure is broken, allowing starch granules to be more accessible within the kernel [34].

1.4.2.A Mechanical Processing of Sorghum

Mechanical processing of sorghum grain is primarily grinding and dry rolling. Grinding sorghum consists of a particle size reduction by using a hammer, a plate mill or a roller mill. Steam rolling is hydrothermal process whereby the grain is processed through a roller mill after being subjected to steam treatment. Through the steam rolling process, fewer fine particles are produced [34]. When sorghum was finely and coarsely ground, the grain was found to have 74% digestibility for cattle for both forms of processing. Similarly [35] observed that sorghum digestibility was 79% digestible for both forms of processing. Johnson et al. (2025) [36] reported that ground grain sorghum had 94% the energy value of dry-rolled corn. While dry rolling increases digestibility compared to unprocessed grain, ground grain sorghum digestibility is still lower than that of ground corn.

1.4.2.B Wet Processing of Sorghum

Wet processing sorghum can consist of harvesting and storing. For early harvesting of the grain the moisture level would be 20-30%, and once harvested the grain typically is stored under anaerobic conditions [34]. Corn and sorghum were harvested at a high moisture content and stored anaerobically; the ME content was determined to be 3.41 for corn and 2.98 Mcal/kg DM for that of sorghum. While this value is numerically different, Owens et al. (1997) [27] determined that the values were not statistically different from each other.

1.4.2.C Hydrothermal Processing of Sorghum

The most common form of heat and moisture processing of sorghum is steam flaking. To steam flake sorghum, grain is exposed to steam for a period of time till the grain is at 18-20% moisture, then passed through corrugated rollers to finish processing [34]. Steam flaking improves the NE by at least 10% when roughage was included in the diet [7]. Chen et al. (1994) [3] determined that the NE_L value for steam flaked corn was 21% greater than that of dry rolled sorghum. It was also determined that there were no differences between the two grains for performance of lactating cows. Steam flaking sorghum is able to make sorghum digestibility more comparable to the digestibility of steam flaked corn. When steam flaking sorghum, bulk density is important for getting the highest digestibility percentage. Differing flaking densities were examined and determined as flaking densities decreased, starch availability increased while there was a decrease in ADG [9]. This decrease in ADG could be due to steers consuming less feed with the lowest flaking density compared to the steers with the highest flaking density.

1.4.3 Sorghum Byproducts

Sorghum grain can be used within numerous different industries. The grain can be ground and used as a human grade gluten free flour, used in ethanol production, as well for in livestock and pet diets. Ethanol production in the United States most commonly uses corn as a feedstock, but sorghum is an important secondary feedstock [37] in the plains states. During the ethanol production process, the starch is largely converted to ethanol and the grain components that remain make up distillers' grains with solubles (DDGS). Compared to corn, sorghum contains a greater amount of fiber and protein and less fat. Consequently sorghum DDGS contain greater amounts of fiber and protein compared to corn DDGS [7]. In a review of sorghum used in dairy cattle diets by Schingoethe et al. (2009) [38], it was reported that when sorghum distillers grains were fed there tended to be less milk produced compared to when corn distillers grains were fed.

The authors concluded from this result that sorghum distillers' grains are less digestible than corn distillers' grains. Al-Suwaiegh et al. (2009) [39], determined that when sorghum distillers' grains are fed to finishing cattle they eat more, but do not gain more than cattle fed corn distillers' grains. Cattle fed corn distillers' grains were 3% more efficient compared to the cattle fed sorghum distillers' grains. This finding concludes the same result that sorghum distillers' grains are less digestible than corn distillers' grains, which corresponds to sorghum grain being less digestible than corn.

1.5 Enzyme Use in Ruminant Diets

Efforts have been made to evaluate different ways to try to increase feed utilization within ruminant diets, one of those methods has been the supplementation of exogenous enzymes. Some studies have reported that supplementation of exogenous enzymes increased weight gain of growing beef cattle and milk production of dairy cows [40]. The improvements in animal performance have been attributed to improvements in ruminal fiber digestion, which led to increases in digestible energy intake. Studies have reported that the greatest response to the fiber-degrading enzymes is in ruminants with compromised fiber digestion [5].

1.5.1 Exogeneous Enzyme Production

Exogeneous enzymes are produced through a batch fermentation process. The batch fermentation process begins with a seed culture. Once the fermentation is complete, the enzyme protein is separated from fermentation residue and the source organism [5]. From there the enzyme is cooled, centrifuged, filtered, and then standardized for quality control [41].

1.5.1 β-mannanase

Mannan is a component of hemicellulose. It is classified into four groups: linear mannan, glucomannan, glactomannan, and galactoglucomannan. Each of the mannan units contains a β-

1,4 linked backbone. The β-1,4-linked backbone contains a mannose sugar or a combination of mannanose and glucose sugars [42]. Beta-mannanase is produced by ruminal bacteria. There are five strains of β-mannanase that degrade more than 20% of the β- mannans within the rumen. Of those five strains, 80% of them are from *Butyrivibrio fibrisolvens.* β-mannanase hydrolyzes the mannosidic bonds of β-mannans, producing mannan-oligosaccharides of varying lengths [43]. When heifers were fed β-mannanase at 600 U/kg of DM, the authors reported an increase in average daily gain (ADG) [44]. The authors concluded that supplementation of β-mannanase at 600 U/kg of diet DM increased growth rate of growing heifers. β-mannanase has also had positive effects on growth and feed efficiency for small ruminants as well. Lee et al. (2014) [45] determined that 480 U/kg of diet DM for growing goats was sufficient to increase growth. β-mannanase has had positive effects on ruminants fed high mannan diets and there have been positive effects for monogastric animals as well.

1.5.2 Fungal Amylase

Fungal amylases have been isolated from several fungal species. Several species of fungal amylases with high amylase activity were isolated from *Aspergillus* sp. [46]. The isolated amylases break down carbohydrates into sugars. Fungal amylases are broken up into three classifications: alpha-amylase, beta-amylase, and gamma-amylase. α-Amylases are dependent upon calcium and are unable to function without the presence of calcium. α-Amylases act randomly along the starch chain, producing maltose, glucose, and “limit dextrin” from amylopectin. β-Amylase acts on the non-reducing end of starch, cleaving off maltose. γ-Amylase cleaves α(1-4) and α(1-6) glycosidic linkages from the non-reducing end of starch producing glucose molecules [46]. In an experiment where fungal amylase was supplemented, starch intake decreased while ruminal starch digestion increased. The authors also reported a decrease in

protozoal numbers. The authors hypothesized that these increases in digestion as well as decrease in protozoal numbers may be indirectly linked. Protozoa limit the rate of starch digestion within the rumen by engulfing starch granules. With the reduction in protozoal numbers there was an increase in rate of starch digestion [47]. Takiya et al. (2017) [48] reported that supplementation of fungal amylases tended to increase dry matter digestibility while having no effect on nutrient intake. The authors originally hypothesized that the addition of amylolytic enzymes would enhance ruminal fermentation by promoting propionate production, the authors reported that there was no change in propionate production with the addition of amylolytic enzymes.

1.5.3 Bacterial Amylase

Starch is hydrolyzed by bacterial amylases and produces products that include dextrin and smaller molecules of differing sizes composed of glucose. Bacterial amylases are subdivided into two groups, endoamylases and exoamylases. Endoamylases are able to randomly hydrolyze linear and branched oligosaccharides of varying lengths from the interior of the starch molecule. Exoamylases hydrolyze short end products from the non-reducing end of the starch molecule. Bacterial amylases have an optimum pH between 6.0 and 7.0 and an optimum temperature range of 25 to 35°C [49]. Studies have reported varying results of bacterial amylase supplementation. When bacterial amylases were supplemented to high and low starch dairy cattle diets, there was tendency to increase starch digestibility [50]. In contrast to the Noziere et al. (2014) [50] experiment, Hristov et al. (2008) [51] reported no effect of enzyme treatments supplemented to dairy cattle on nutrient digestion. Varying results with enzyme supplementation could be due to several factors. One factor affecting enzyme activity could be a rumen pH that is outside the optimum range of the bacterial amylase. Both studies mentioned previously utilized dairy cattle, where the diets have lower levels of starch inclusion compared to finishing diets. When fed to

finishing cattle, bacterial amylases could have differing results. One option for amylase inclusion in finishing diets is to utilize high-amylase corn. When high-amylase corn was fed to finishing cattle an increase in starch availability along with increased microbial digestion was reported [52].

1.5.4 Xylanase

Xylanases are produced by several sources, but the main sources of xylanase production are fungi and bacteria. The optimal pH of bacterial xylanases is 5 to 9 and the optimal temperature is between 35 and 60°C. Endoxylanases are important in the breakdown of the xylan molecule into pentose units. Endoxylanases are able to cleave glycosidic bonds creating xylooligosaccharides [53]. Exoxylanases are able to hydrolyze xylan and xylooligosaccharides from the non-reducing end, producing short chain units [54]. As mentioned previously, the use of exogeneous enzymes has had differing responses. Pech-Cervantes et al. (2021) [55] examined the use of a xylanase enzyme on milk production. There was no difference in milk yield for the cattle fed the xylanase enzyme compared to the control fed cattle. There was an increase in the protein percent within the milk for the cattle fed the xylanase enzyme. A study utilizing Holstein cows reported a decrease in silage digestibility and no difference in starch digestion when supplemented at 1.5g xylanase per kg of DM [56]. While no difference in starch digestion was reported, there was a difference in silage type starch digestibility between corn and sorghum silage. The authors attributed this difference to there being a greater amount of starch within corn silage compared to that of sorghum silage. Xylanases not only have the potential to be beneficial in dairy cattle diets, but also in beef cattle diets. McAllister et al. (1999) [57] performed a backgrounding study that reported a tendency to have an increase in final weight when supplemented with xylanase. The study also reported that there was no difference in ADG.

1.5.5 Cellulase

Cellulases are cellulose degrading enzymes [58]. Cellulases are able to degrade cellulose through a complex of enzymes such as endoglucanase, exoglucanase, and β-glucosidase. Endoglucanase is able to produce varying sizes of oligomers by hydrolyzing cellulose chains at random. Exogluanases are able to produce cellobiose by hydrolyzing the cellulose chain from the non-reducing end. β-Glucosidase produce cellobiose and glucose by hydrolyzing short chain cellulose oligomers [5]. A study was conducted to evaluate a xylanase and cellulase mixture applied to silage. The authors reported no difference in DM digestibility between the enzyme dosed silage and the control silage, as well as no difference between cellulolytic bacterial numbers within the rumen [57]. Titi et al. (2004) [59] reported an increase in NDF digestibility for barley silage treated with cellulase enzymes compared to nontreated silage. Within the same study the authors reported that there was an increase in final body weight for the calves fed the enzyme treated silage. This increase in final body weight could be attributed to an increase in digestibility. When a cellulase and xylanase blend was fed to dairy cattle there was no difference in milk components, yield, or body weight gain. These results indicate that feeding the cellulase and xylanase blend did not improve digestion or performance of the dairy cattle during the trial [60].

1.5.6 Protease

Proteins are comprised of amino acids. Amino acids primarily consist of an amino and carboxy group. There are three primary groups of amino acids: hydrophobic, hydrophilic, and aromatic. Protease enzymes are able to degrade proteins by hydrolyzing bonds before and after specified amino acids [61]. Endogenous protease enzymes have been heavily studied within human models. Exogenous enzymes have not been as heavily researched in ruminants; there have been a

few studies supplementing proteases to pigs. The monogastric digestion process is different than ruminants. Ruminants utilize enzymatic hydrolysis and microbial fermentation, as well as mastication. Ruminants utilize mastication to increase saliva production, which optimizes digestion by helping to buffer the rumen environment. Monogastric animals utilize enzymatic digestion to break down feed particles. Mastication within the monogastric digestive system is utilized first to break down particle sizes along with adding digestive enzymes through the addition of saliva [62–64]. The process of breaking down amino acids is similar between the two. In a study where a neutral protease, an acid protease, and a combination of both proteases were fed to chickens, the researchers reported no difference in body weight gain, but there was a numerical improvement in final body weight. The authors also reported no difference in breast meat yield [65]. In another study utilizing chickens, researchers reported an increase in apparent metabolizable energy (AME) in the protease supplemented group compared to the non-enzyme supplemented group [66]. Researchers who examined effects of supplementing protease enzyme to growing piglets fed low protein diets observed a tendency to increase nitrogen retention, leading to an increase in piglet performance [67]. In a growing-finishing pig trial in which protease enzymes were supplemented, the researchers observed no differences in carcass characteristics. The authors did report an improved growth rate for the pigs [68]. Vera et al. (2012) [6] examined effects of protease enzyme supplementation to growing and finishing steers. During the growing phase, the authors reported subtle but non-significant differences in nutrient digestibility. During the finishing phase, the authors reported an increase in DM, acid detergent fiber (ADF), nitrogen, and NDF digestibility when steers were supplemented exogenous protease enzymes. Colomabtto et al. (2009) [69] reported an increase in IVDMD when rolled corn was treated with exogenous protease enzymes. Eun and Beauchemin (2007) [70] reported a decrease

in diet intake, with an increase in total tract digestibility for DM, OM, nitrogen, and ADF when exogeneous protease enzymes to dairy cattle. In the second part of the study a complementary *in vitro* study was performed, which demonstrated an increase in gas production when exogeneous protease enzymes were added to the diet, but no effects on ADF degradability; however, DM and NDF degradability were increased with enzyme supplementation. Sucu et al. (2014) [71] reported when dairy cattle were supplemented exogeneous protease enzymes there was a decrease in DMI in the enzyme fed cattle with no significant differences in milk composition. While protease enzymes have not been studied extensively, reports have had differing results for DMI and nutrient digestibility.

1.6 Future Research

There have been many articles published evaluating the effects fiber degrading enzymes within ruminant diets, but there are some gaps when looking into supplementation of protease enzymes. Future research that could be beneficial would be examining the effects of protease enzymes in dairy as well as beef cattle diets. Future research could also examine differing processing methods. The utilization of different pH optimum enzymes within dairy and beef cattle diets, is beneficial to examine what enzyme would have the most beneficial results.

1.7 Conclusion

With the growing human population and increases in the amounts of milk and meat consumed, along with decreased water within the Ogallala Aquifer, there is a need to find alternative drought-resistant feedstuffs. Sorghum grain could be an alternative feedstuff. Sorghum is more drought-resistant than corn is, and through grain processing sorghum could have starch digestibility similar to that of corn. Within the cattle feeding industry sorghum is an underutilized feedstuff [72]. Along with processing, protease enzymes have posed promising

results with increases in digestion along with tendencies to increase ADG [6]. These findings indicate that there is potential for protease enzymes to have beneficial increases in sorghum digestibility, as well as provides groundwork for research in optimizing sorghum digestibility within finishing beef cattle diets.

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Chapter 2 - Effects of exogeneous enzymes on performance of finishing cattle fed sorghum-based diets

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Simple Summary

This experiment was conducted to examine effects of an exogeneous enzyme blend on nutrient digestibility, feedlot performance, and carcass characteristics of steers fed a sorghum-based finishing diet. Pyramid PowerGrow Ruminant was supplemented at a rate of 750 mg/kg of feed (86% dry matter basis). There were subtle but non-significant improvements in nutrient digestibility, feedlot performance, and carcass characteristics with enzyme supplementation.

Abstract

The objective of this study was to investigate effects of an exogeneous enzyme blend on nutrient digestibility, feedlot performance, and carcass characteristics of crossbred steers (n=280; 441.74 kg ± 15.19 kg initial body weight) fed sorghum-based finishing diets. Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, KS) was supplemented at a rate of 750 mg/kg of feed (86% dry matter basis) fed sorghum-based finishing diets. Diets consisted of 59.62% ground sorghum, 25% dried sorghum distillers grains with solubles, 12% corn silage, and 3.35% supplement, and were fed once daily *ad libitum*. Fecal samples were collected on day 32 and 67 to determine nutrient digestibility. After 148 days on feed steers were harvested, and carcass characteristics were recorded. There were subtle but non-significant improvements in nutrient digestibility, feedlot performance, and carcass characteristics with enzyme supplementation. Fecal pH was impacted by sampling day, day 32 had higher fecal pH than the day 67 samples. Numerical decreases in fecal volatile fatty acid concentrations were observed in the enzyme treatment compared to the control treatment. Numerical increase in final body weight were observed in the enzyme treatment compared to the control treatment.

Keywords: sorghum, digestibility, enzymes, feedlot

Abbreviations

Neutral detergent fiber (NDF), Dry matter (DM), Average daily gain (ADG), Feed efficiency (G:F), Acid detergent insoluble ash (ADIA), organic matter (OM), dried distillers grains with solubles (DDGS), days on feed (DOF), hot carcass weight (HCW), United States Department of Agriculture (USDA), body weight (BW), Deionized (DI), dry matter intake (DMI), volatile fatty acid (VFA); Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

2.1 Introduction

The Great Plains region produces a major portion of finished cattle in the U.S. [1]. Within eight states (South Dakota, Wyoming, Colorado, Kansas, Nebraska, New Mexico, Oklahoma, and Texas) in the Great Plains region spanning 450,000 km^2 is the Ogallala Aquifer. The Ogallala Aquifer provides 30% of irrigation water and 70% of the ground water in the US [2]. Irrigation within the High Plains has contributed to significant depletion of the aquifer [3], as water is being withdrawn from the aquifer at a rate that exceeds its natural ability to recharge [4]. The importance of water conservation efforts thus has become more widely recognized. Within the Great Plains region, predominant irrigated crops are corn and alfalfa. Corn has a minimum water requirement of 277 mm [5], while alfalfa has a water requirement between 800 and 1600 mm depending on the climate and growing period [6]. One option to decrease water usage is to cultivate sorghum, which has requires less water than corn or alfalfa. Sorghum requires a minimum of 175 mm of water [5]. This advantage in semi-arid regions has led sorghum to be the fifth most produced cereal grain globally [7]. Sorghum has, however, been found to be less ruminally digestible than corn, and thus has been less desirable for producers to use [8]. Protein within the sorghum grain forms complexes around the starch granules that inhibit the ability for the protein and starch to be degraded [9–11]. Feeding exogenous enzymes has the potential to mitigate limitations to starch and protein degradation within sorghum. The use of exogenous enzymes has yielded varying responses, and not all studies report improvements in animal performance [12]. He et al. (2013) [13] reported an increase in wheat dried distillers' grains with solubles degradation in response to supplementation of exogenous enzymes. There has been limited research utilizing exogenous enzymes to increase sorghum digestibility within sorghum-based finishing diets.

We hypothesized that supplementing a blend of exogeneous enzymes (Pyramid PowerGrow Ruminant; Scott City, KS) would enhance sorghum digestibility thereby improving feedlot performance carcass characteristics.

2.2 Materials and Methods

2.2.1 Experimental Design and Treatments

Crossbred steers (n=280, 441.74 kg ± 15.19 kg) were utilized to evaluate the effects of feeding exogeneous enzymes on nutrient digestibility, feedlot performance, and carcass traits in steers fed sorghum-based finishing diets. During the experiment three steers died, two of non-apparent causes, the third was physically injured and later died from the injury. The experiment was a randomized complete block design with two treatments. There were 18 blocks, and treatments were randomly assigned to pen within a block. Blocks were determined by chute order, sorting two pens at a time randomly. Treatments consisted of a control diet (Control) without added enzymes, and a diet with Pyramid Powergrow Ruminant included at the rate of 750 mg/kg feed on an 86% DM basis. The experiment was conducted at the Southeast Research and Extension Center, Mound Valley, KS.

2.2.2 Initial Processing

Upon arrival at the feedlot steers were allowed *ad libium* access to Fescue hay and water. Steers were individually weighed and vaccinated for respiratory diseases with Pyramid 5 + Presponse SQ (Boehringer Ingelheim, Duluth, GA), clostridial diseases with Bovilis Covexin 8 (Merck Animal Health, Rahway, NJ). Steers received the injectable antiparasitic Cydectin (Elanco Animal Health, Indianapolis, IN), and received an oral drench of Lactipro (Axiota Animal Health; Fort Collins, CO). Steers were implanted with Snyovex One Feedlot (Zoetis Animal

Health, Florham Park, NJ), and administered Draxxin (Zoetis Animal Health, Florham Park, NJ) as a prophylactic. Uniquely numbered ear and radio frequency identification tags (Allflex, Rahway, NJ) were used to identify each animal.

2.2.3 Finishing Diets and Preparation

Diets are shown in table 2-1. The finishing diets consisted of 59.62% ground grain sorghum, 25% sorghum-based dried distillers' grains with solubles (DDGS), 12% corn silage, and 3.38% supplement. For the enzyme treatment, Pyramid PowerGrow Ruminant was added to the supplement (replacing ground corn) to provide 750 mg/kg (86% dry matter basis) in the complete diet. The last 33 days on feed (DOF) Optaflexx 500 (Elanco Animal Health, Indianapolis, IN) was fed to the steers at a rate of 25 mg/kg daily. Sorghum grain was processed to a particle size of 944 µm, and DDGS had a mean geometric particle size of 464 µm. Particle size was determined using a 13-sieve stack (3,360 µm > 2,380 µm > 1,680 µm > 1,190 µm > 841 µm > 595 µm > 420 µm > 297 µm > 210 µm > 149 µm > 105 µm > 74 µm > 53 µm and a pan); 100 grams of sample were placed onto the top sieve along with 0.5 grams of sieve agent (Gilson Company inc., Lancaster, OH), and placed into the Ro-Tap shaker (W.S. Tyler, Mentor, OH) for 10 minutes. Particles collected each sieve were weighed and recorded and mean geometric particle size was then calculated as described by Wilcox et al. (1970) [14]. Total mixed rations were fed in the morning throughout experiment. The adaptation phase of the experiment consisted of three intermediate diet step-ups; each being fed for seven days. Each morning bunks were assessed to determine the amount of feed to deliver for *ad libitum* intake, rejected feed samples were collected on Monday and Thursday each week. Ingredients were sampled weekly and dried in a 55°C oven for 48 hours for DM determination. Dry matter percent of rejected feed samples were determined by weighing a 100 g subsample and drying at 105°C for 24 hours.

2.2.4 Body Weights and Harvesting

Cattle were shipped to a commercial abattoir in Western Kansas after 148 DOF. Hot carcass weight (HCW) and liver abscess scores were determined at the time of harvest, and after a refrigeration period of 96-h, United States Department of Agriculture (USDA) yield grade and quality grades; ribeye area; 12th- rib subcutaneous fat thickness; marbling scores; and percent kidney, pelvic, and heart fat were obtained using camera images (VBG 2000, E+V Technology GmbH & Co. KG, Oranienburg, Germany).

Liver scoring followed the Elanco scoring system (None = no abscess; A- = one – two small abscesses; A = two – 4 well-defined abscesses; A+ = more than 4 active and large abscesses) (Liver Abscess Technical Information AI 6288; Elanco Animal Health, Greenfield, IN)

Marbling scores were assessed as detailed by USDA: (200-299 = trace marbling; 300-399 = slight marbling; 400-499 = small amounts of marbling; 500-599 = modest amounts of marbling; 600-699 = moderate amounts of marbling; 700-799 = slightly abundant amounts of marbling; 800-899 = moderately abundant marbling).

Final body weight (BW) was determined by the formula:

$$Final\ BW = \frac{HCW}{0.635}$$

2.2.5 Laboratory Analyses

Fecal samples were collected on February 16, 2025, and March 24, 2025. Fecal pH was determined by collecting freshly voided fecal pats from the pen surface (approximately 10/pen), placed into plastic bags, sealed, and stored on ice. Approximately 3 hours after collection, samples were thoroughly homogenized. A 25-g subsample was blended with 50 mL deionized

(DI) water for 2 minutes and pH was recorded with a portable pH meter (Thermo Scientific Orion Star A Series; Thermo-Fischer, Waltham, MA). Fecal VFA were determined by combining a 10 g subsample with 20 mL of DI water into a 50-mL polycarbonate centrifuge tube (Fischer Scientific, Hampton, New Hampshire). Fecal matter and DI water were allowed to thoroughly mix in a controlled environment incubator shaker (New Brunswick Scientific, New Brunswick, New Jersey) for 20 minutes at 50 rpm. A 1-mL subsample was taken from the centrifuge tube and mixed with 0.25 mL of 25% m-phosphoric acid and frozen at -20°C. Liquid extract for VFA analysis was centrifuged at 14,000 rpm (Centrifuge 5415 C; Eppendorf, Hamburg, Germany). Once centrifuged, 0.4 mL of supernatant was transferred to a chromatography vial, and 1.2 mL of pivalic acid was added to the vial as the internal standard. Concentrations of VFA were determined using an Agilent 7890A GC system (Agilent Technologies; Santa Clara, California) equipped with a flame ionization detector and capillary column (DB-FATWAX UI 20 m x 0.180 mm x 0.18 μm film thickness; Agilent Technologies, Santa Clara, California). Fecal samples and ingredient samples were weighed and dried at 55°C until completely dry, and weights were taken again to determine dry matter percent. Dried samples were ground through a 1-mm screen in a Wiley Mill grinder (Model 4; Thomas Scientific, Swedesburo, NJ). Samples for nutrient digestibility were analyzed by SDK Labratories (Huchinson, KS). Acid detergent insoluble ash was utilized as an internal marker for estimation of apparent total tract nutrient digestibility [15]. Fecal samples from day 67 were utilized. By day 67 the assumption was made that steers would be fully adapted to the finishing diets. The formula used [16]:

$$Digestibility\ \% = 100 - [100\ x\ \frac{acid\ detergent\ insoluble\ ash\ in\ feed}{acid\ detergent\ Insoluble\ ash\ in\ feces}\ x\ \frac{Nutrient\ concentration\ in\ feed}{Nutrient\ concentration\ in\ feces}]$$

2.2.6 Statistical Analysis

Data were analyzed using the GLIMMIX procedure of the Statistical Analysis System (SAS 9.4; SAS Studio, Cary, NC). Fixed effect was treatment (Control and enzyme), experimental unit was the feedlot pen, and random effect was block. Statistical tendencies were determined at $0.05 < P < 0.10$, and statistical significance was declared at $P \leq 0.05$. Initial body weight was used as a covariate to evaluate incidence of liver abscesses, USDA quality grade, USDA yield grade, hot carcass weight, final weight, marbling score, 12^{th} rib subcutaneous fat, kidney, pelvic, and heart fat percent, ribeye area, and dressing yield percentage. Least-squares means were separated using the pdiff function to evaluate differences between treatments.

2.3 Results and Discussion

During the duration of the study three steers were removed. Two died for undetermined reason from the enzyme treatment, another from the control treatment after sustaining a physical injury. We observed that the addition of exogeneous enzymes (Pyramid PowerGrow Ruminant; Scott City, KS) resulted in subtle but not significant differences in nutrient digestibility, feedlot performance, and carcass characteristics among the treatment groups.

There were subtle but non-significant differences between treatments for fecal pH and VFA concentrations. Fecal VFA and pH data are depicted in Table 2-2. Wheeler et al. (1977) and Hoover et al. (1978) [17,18] reported that in cattle fed high concentrate diets low fecal pH and greater amounts of starch in the feces are correlated. The lack of differences in starch digestibility corresponded to the lack of differences in fecal pH. Acetate, propionate, butyrate, and total VFA concentrations were numerically increases in the control treatment compared with the enzyme treatment, although the differences were not statistically significant. These results

represent that there was a similar amount of nutrients reaching the hindgut, and is expected due to similar DMI as well as nutrient digestibility between the two treatment groups.

No statistical differences were observed between treatment groups for nutrient digestibility ($P > 0.10$), with results shown in Table 2-3. Contrary to the initial hypothesis, dry matter, protein, nor starch digestibility were improved with enzyme application. Vera et al. (2012) [19] reported an increase in DM, nitrogen, and NDF digestibility when protease enzymes were supplemented during the finishing phase. When lactating dairy cattle were fed exogenous proteolytic enzymes there was no difference between starch digestibility, but a decrease in starch intake in the enzyme supplemented group was reported [20]. Although starch digestibility results in the two referenced studies were comparable to those observed in the present study, it is important to recognize that grain digestibility may differ, as barley is generally considered more rapidly digestible than sorghum [21].

Enzyme supplementation produced numerical but not significant differences in final BW, ADG, DMI, and G:F ($P > 0.10$). Performance data are depicted in Table 2-4. Comparable dry matter intake and average daily gain for the treatments was accompanied by the absence of differences in nutrient digestibility and final BW. Final body weight had a numerical but non-significant differences between the enzyme and control treatments; the enzyme treatment had a 5.4 kg increase in final BW. While pigs are monogastric animals, numerous well-documented studies have demonstrated the effectiveness of enzyme supplementation on animal performance. Studies observed [22,23] increased body weight and ADG when weaned pigs were supplemented an enzyme blend. Monogastric animals exhibit hindgut fermentation, with enzymatic digestion being utilized within the stomach of the animal [24]. Monogastric animals are unable to utilize fibrous plant material such as cellulose [25]. Ruminants on the other hand utilize enzymes and

microbes within the rumen to ferment feedstuffs within the rumen to nutrients that are then able to be utilized and absorbed by the animal [26]. While the process of digestion is different among monogastric and ruminants, the basics of how protease enzymes function is similar. When protease enzymes are supplemented, they degrade proteins by cleaving proteins via hydrolysis at specific bonds between specified amino acids [27]. Vera et al. (2012) [19] reported similar results with no difference in DMI or G:F. The authors reported a tendency for improved ADG in the enzyme treatment compared to the control. Figure 2-1 depicts DMI during the 148-d trial. Dry matter intake reached the highest value during week 14, then decreased. Lewis et al. (2020) [28] reported on the relationship between liveweight and intake. Intake was at the greatest before cattle reached harvest weight. In the present study, DMI increased in week 17 after the decrease in weeks 14 and 15.

Limited research has been reported on the effects of proteolytic enzyme addition on carcass characteristics. Beauchemin et al. (1997) [29] reported that enzyme addition influences nutrient digestibility and cattle growth, but carcass characteristics were only indirectly impacted by enzyme addition. In the current study, no differences in carcass characteristics (Table 2-5) were observed ($P > 0.10$). Given that DMI, ADG, and G:F were similar between treatments, minimal differences in carcass characteristics between the control and enzyme treatments were expected. Zobell er al. (2000) [30] reported similar results with no differences in carcass characteristics when enzymes were supplemented to finishing steers on a barley based diet. Vera et al. (2012) [19] observed similar results for HCW and marbling scores but reported a tendency for the enzyme fed cattle to have a greater ribeye area compared to steers fed diets without added enzyme.

The primary determining factor for quality grade is marbling score [31]. Considering there were no detectable differences in marbling scores between the two treatments, it is expected that there are non-significant differences between quality grades (Table 2-7). Moon et al. (2003) reported numerous carcass characteristics impact yield grade. Among carcass traits, the greatest negative impact on yield grade was backfat thickness. Carcasses that had larger ribeye areas and reduced backfat are associated with improved yield grades. Yield grade is nonlinearly related to slaughter weight. As previously noted, carcass characteristics did not differ ($P > 0.10$), and therefore differences in yield grade (Table 2-8) were not anticipated.

Future research should expand upon the finding reported, and examine differing supplementation rates of exogeneous enzymes, and examine supplementing proteolytic enzymes from differing fungal and bacterial sources, along with different pH minimums and maximums.

2.4 Conclusion

Results from this experiment indicate that Pyramid PowerGrow Ruminant had modest but non-significant effects on nutrient digestion, feedlot performance, and carcass characteristics.

Conflict of Interest

The authors declare no conflicts of interest.

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Author Contributions

Conceptualization, J.D.; methodology, J.D.; validation, J.D. and E.K.; formal analysis, J.D.; investigation, J.D. and E.K.; data curation, J.D. and E.K.; writing—original draft preparation, E.K.; writing—review and editing, J.D., L.M., J.F., and S.S.; visualization, J.D.; supervision, J.D.; project administration, J.D.; funding acquisition J.D.

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Data Availability Statement

The raw data supporting conclusions of this article will be available by the authors upon request.

Institutional Review Board Statement

Ethics Approval – Procedures for this experiment were approved by the Kansas State University Institutional Animal Care and Use Committee, license number 4760.

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Table 2-1: Composition of finishing diets (% of DM) containing either the enzyme blend supplement or the ground corn supplement.

Item	Diet
Ingredient, %	
Ground sorghum	59.62
Dried sorghum distillers grains with solubles	25.00
Corn silage	12.00
Supplement [1,2]	3.38
Particle Size, µm	
Ground sorghum	944
Dried sorghum distillers grains with solubles	464
Analyzed nutrient composition[3], %	
Crude protein	18.85
Neutral detergent fiber	19.44
Organic matter	95.61
Starch	43.20

[1]Enzyme supplement includes Pyramid Powergrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Supplement was formulated to provide on a dry matter basis; 2,205 IU/kg of vitamin A**;** 15.43 IU/kg of vitamin E; 0.7% calcium, 0.33% phosphorus, 0.25% salt, 10 mg/kg of copper; 40 mg/kg of zinc; 0.50 mg/kg of iodine; 0.10 mg/kg of cobalt; 0.10 mg/kg of selenium; 40 mg/kg of manganese; 33 mg/kg of monensin; 7 mg/kg of tylosin; 25 mg/kg daily of ractopamine hydrochloride the last 33 DOF.

[3]Analyzed by SDK Laboratories (Huchinson, KS)

[4]Particle size was determined using a 13-sieve stack according to sizes (3,360 µm > 2,380 µm > 1,680 µm > 1,190 µm > 841 µm > 595 µm > 420 µm > 297 µm > 210 µm > 149 µm > 105 µm > 74 µm > 53 µm and a pan); 100 grams of sample were put onto the top sieve along with 0.5 grams of sieve agent (Gilson Company inc., Lancaster, OH), and placed into the Ro-Tap shaker (W.S. Tyler, Mentor, OH) for 10 minutes. Particles collected from each sieve were weighed and recorded. Total mixed rations were fed in the morning throughout experiment.

Table 2-2: Effects of an enzyme blend on VFA concentrations and pH of feces (wet basis) from feedlot steers fed sorghum-based diets with or without exogeneous enzymes.

				P-Value		
Item	Control	Enzyme[1]	SEM[2]	Treatment	Day	T*D[5]
Fecal dry matter, %	26.29	26.51	0.301	0.583	<0.001	0.002
pH[3]	6.0	6.0	0.04	0.548	0.074	0.150
Acetate, mM[4]	44.4	41.0	2.50	0.328	0.588	0.779
Propionate, mM[4]	21.4	19.0	1.34	0.218	0.863	0.692
Butyrate, mM[4]	10.0	9.0	0.77	0.359	0.905	0.900
Total VFA, mM	75.8	68.9	4.42	0.280	0.738	0.765
A:P	2.2	2.2	0.068	0.597	0.264	0.402

[1]Pyramid Powergrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

[3] Freshly voided fecal pats were collected from the pen surface (approximately 10/pen), placed into plastic bags, sealed, and stored on ice. Approximately 3 hours after collection, samples were thoroughly homogenized, a 25-g subsample was blended in 50 mL of deionized water for 2 minutes, and pH was recorded with a portable pH meter (Thermo Orion Star A Series; Thermo-Fischer, Waltham, MA).

[4]Fecal VFA were determined by combining 10 g of sample with 20 mL of DI water into a 50-mL polycarbonate centrifuge tubes (Fischer Scientific, Hampton, New Hampshire). Fecal matter and DI water were allowed to thoroughly mix in a controlled environment incubator shaker (New Brunswick Scientific, New Brunswick, New Jersey) for 20 minutes at 50 rpm. A 1 mL subsample was taken from the centrifuge tube and mixed with 0.25 mL of 25% m-phosphoric acid and frozen at -20°C for analysis. Liquid for VFA analysis was centrifuged at 14,000 rpm (Centrifuge 5415 C; Eppendorf, Hamburg, Germany). Once centrifuged, 0.4 mL of supernatant was then transferred to chromatography vials, 1.2 mL of pivalic acid solution was added to the vials as the internal standard. Concentrations of VFA were determined using an Agilent 7890A GC system (Agilent Technologies; Santa Clara, California) equipped with a flame ionization detector and capillary column (DB-FATWAX UI 20 m x 0.180 mm x 0.18 µm film thickness; Agilent Technologies, Santa Clara, California).

[5]Treatment by day interaction.

Table 2-3: Effect of an enzyme blend on apparent total tract nutrient digestibility when added to sorghum-based diets for feedlot steers.

Digestibility[3], %	Control	Enzyme[1]	SEM[2]	*P*-Value
Dry matter	72.75	74.25	1.626	0.444
Organic matter	74.11	75.46	1.614	0.490
Starch	89.58	91.26	1.078	0.150
Protein	69.70	70.73	1.910	0.668
Neutral detergent fiber	64.39	65.09	1.952	0.765

[1]Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

[3]Samples were analyzed by SDK Laboratories (Huchinson, KS).

Table 2-4: Effects of an enzyme blend on performance of feedlot steers fed sorghum-based diets with or without exogenous enzymes.

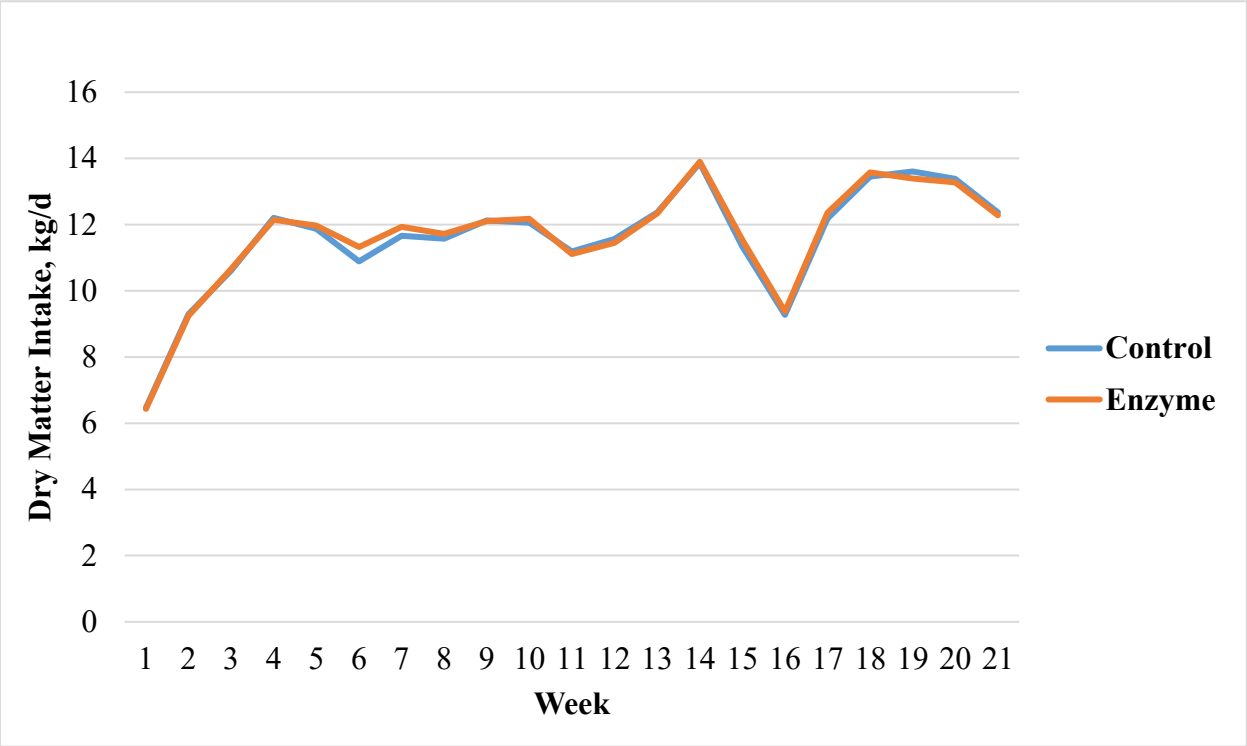
Item	Control	Enzyme[1]	SEM[2]	*P*-value
Initial body weight, kg	441.5	442.6	7.90	0.808
Final body weight, kg[3]	609.2	614.6	7.66	0.341
Dry matter intake, kg/d	11.59	11.62	0.255	0.831
Average daily gain, kg	1.13	1.17	0.052	0.341
Gain:feed	0.098	0.100	0.0018	0.276

[1]Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

[3]Final body weight (BW) was determined by the formula: $Final\ BW = \frac{Hot\ Carcass\ Weight}{0.635}$

Figure 2-1: Dry matter intake by steers fed sorghum-based diets with or without exogenous enzymes.[1,2]



[1]Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Effect of diet*week; ($P > 0.10$). effect of diet: ($P > 0.10$); effect of week: ($P < 0.01$);

Table 2-5: Effects of an enzyme blend on carcass characteristics of feedlot steers fed sorghum-based diets with or without exogeneous enzymes.

Item	Control	Enzyme[1]	SEM[2]	*P*-value
Hot carcass weight, kg	387.0	390.2	4.87	0.341
Longissimus muscle area, cm^2	84.6	86.0	0.12	0.140
12^{th} - rib subcutaneous fat thickness, cm	1.19	1.21	0.016	0.797
Marbling score[3]	568	552	12.3	0.316
Kidney, pelvic, and heart fat, %	1.84	1.82	0.015	0.465

[1]Pyramid PowerGrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

[3]Marbling score: 400 to 499 = small degree of marbling; 500 to 599 = modest degree of marbling.

Table 2-6: Effects of an enzyme blend on incidence of liver abscesses in feedlot steers fed sorghum-based diets.

Item[2]	Control	Enzyme[1]	SEM[3]	*P*-value
Total abscessed livers, %	10.5	15.4	2.89	0.243
None, %	89.5	84.7	2.89	0.538
A-, %	4.9	8.9	1.75	0.119
A, %	2.8	4.4	1.62	0.503
A+, %	2.8	2.2	1.24	0.723

[1]Pyramid Powergrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Elanco scoring system (Elanco US, Inc., Greenfield, IN): 0 for no abscess; A- for one or two small abscesses or inactive scars; A for one or two large abscesses or several small abscesses; A+ for multiple large abscesses.

[3]Standard error of the mean.

Table 2-7: Effects on USDA Quality Grade of feedlot steers fed sorghum-based diets without exogeneous enzymes.

USDA Quality Grade, %	Control	Enzyme[1]	SEM[2]	*P*-value
Prime	24.3	22.9	4.25	0.793
Premium Choice[3]	40.2	38.3	4.42	0.764
Low Choice	28.1	30.3	4.12	0.710
Select	6.4	8.1	1.97	0.512

[1]Pyramid Powergrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

[3]Represents the upper 2/3^{rds} of the choice grade, which is consistent with modest and moderate amounts of marbling.

Table 2-8: Effects of an enzyme blend on USDA Yield Grade of feedlot steers fed sorghum-bases diets with or without exogeneous enzymes.

USDA Yield Grade, %	Control	Enzyme[1]	SEM[2]	*P*-value
Yield grade 1	2.1	0.7	1.20	0.424
Yield grade 2	37.4	40.0	4.18	0.657
Yield grade 3	53.1	52.1	4.16	0.854
Yield grade 4	7.4	6.5	1.79	0.700
Yield grade 5	0.0	0.7	0.50	0.328

[1]Pyramid Powergrow Ruminant (Pyramid Feeds, Scott City, Kansas) in lieu of ground corn at a rate of 750 mg/kg of feed (86% dry matter basis). Enzyme blend consisted of the following components: 5,000 HCU/g beta mannanase; 1200 SKB/g of fungal amylase; 600 BAU/g of bacterial amylase; 250 XU/g of xylanase; 500 HUT/g of fungal protease; 800 PC/g of neutral protease; 1100 CU/g of cellulase (values were determined from product specification document provided by Pyramid Feeds). Abbreviations: Hemicellulose unit (HCU); alpha-amylase dextrinizing unit (SKB); bacterial amylase unit (BAU); xylanase unit (XU); cellulase unit (CU); bacterial protease unit (PC); hemoglobin units on the tyrosine basis (HUT).

[2]Standard error of the mean.

Chapter 3 - Effects of exogeneous enzymes on *in vitro* digestion of cereal grains

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Simple Summary

The purpose of this experiment was to examine effects of four exogenous enzymes on *in vitro* digestion of cereal grains. Enzymes were added at a rate of 3 or 30 μL per 50-mL culture tube. When enzymes were added to the culture there were subtle differences between the control and enzyme supplemented culture. There were no effects of exogeneous enzyme addition on *in vitro* dry matter digestibility or total VFA production. There was a tendency for increased isovalerate production and methane production per gram of substrate digested when exogeneous enzymes were supplemented.

Abstract

The objective of this experiment was to examine four protease enzymes for their effect on terminal pH, *in vitro* dry matter digestibility (IVDMD), and volatile fatty acid production when added to in vitro cultures containing cereal as substrates. There were four proteolytic enzymes evaluated: fungal acid protease, fungal protease, fungal neutral protease, Liquipanol, along with control corn and sorghum treatments, and a blank. Each of the four enzymes were added to cultures at concentration of 30 μL or 3 μL per 50-mL tube using ground grain sorghum as the substrate. Additionally control treatments consisting of corn or ground grain sorghum without added enzyme were used. Two runs were performed for this experiment, run one contained 0.3 mg/ tube of substrate and run two used 0.5 mg/ tube of substrate dry matter. The control corn treatment has lower terminal pH, greater IVDMD percent, and greater volatile fatty acid production ($P < 0.01$). There were small but non-significant effects of enzyme addition to sorghum for terminal pH of cultures, IVDMD, and total VFA production ($P > 0.10$). Isovalerate production tended to increase with exogeneous enzyme addition. High rates of enzyme addition decreased IVDMD, and tended to decrease total VFA production.

Key words: *In Vitro,* Sorghum, Protease

Abbreviations

In vitro dry matter digestibility (IVDMD), volatile fatty acids (VFA), acetate:propionate ratio (A:P), branched chain fatty acid (BCVFA), organic matter (OM), dry matter (DM), methane production per gram of substrate digested (methane/g; CH_4/g), acetate (A), propionate (P), isobutyrate (I), Butyrate (B), valerate (V), isovalerate (IV), total VFA (VFA)

3.1 Introduction

In January 2025, 14.3 million cattle on feed in the United States [1]. The U.S. the High Plains region is home to a majority of cattle on feed in the U.S. [1]. The High Plains region is underpinned by the Ogallala Aquifer which spans approximately 450,000 km^2 [2]. This aquifer accounts for 70% of the ground water and 30% of irrigation water in the U.S. [3]. Within Kansas, 14% of cropland is irrigated. Recharge rates of the aquifer vary throughout the High Plains region [4], but the necessity for water conservation efforts is increasingly recognized. At the current rates of depletion, agricultural irrigation may be un able to be supported by the aquifer by 2100 [5]. Corn has a minimum water requirement of 277 mm [6], while alfalfa has a water requirement between 800 and 1600 mm depending on the climate and growing period [7]. One option for water conservation is to plant sorghum. Sorghum is an important cereal crop that can be grown in arid and semi-arid regions. Due to sorghum's lower water requirement, the plant is able to withstand periods of water stress [8]. While sorghum can be utilized as a water conservating crop, it generally is regarded as being inferior to corn as a livestock feed. Starch and protein interactions within the endosperm of the grain limits starch degradation [9,10]. He et al. (2013) [11] reported an increase in wheat DDGS degradation with the supplementation of exogenous enzymes. There is limited research utilizing exogenous enzymes to increase sorghum digestibility within sorghum-based finishing diets.

We hypothesized that protease addition to *in vitro* cultures would increase digestibility of sorghum grain. This experiment was conducted to evaluate the impacts of fungal acid proteases, fungal proteases, fungal neutral proteases, and liquipanol on *in vitro* sorghum digestion.

3.2 Materials and Methods

3.2.1 Experimental Design

Corn and sorghum samples were processed using a cyclone sample mill (Udy Corporation, Fort Collins, Colorado) equipped with a 1-mm screen. Processed grains were placed in plastic bags and stored at -20°C. To determine dry matter percent of each sample, a 5-gram aliquot was weighed (in duplicate) and dried at 105°C for 24 h. Each grain was evaluated using an *in vitro* dry matter disappearance assay. Processed grain was weighed (0.3 g/ tube of substrate DM for run 1 and 0.5 g/ tube of substrate DM for run 2) into 99 individual 50-mL polycarbonate centrifuge tubes (Fischer Scientific, Hampton, New Hampshire). There were 9 replicates, enzymes were added to the *in vitro* culture at a rate of 30 μL/ 50-mL tube for the high inclusion rate and 3 μL/ 50-mL tube for the low inclusion rate. The enzymes utilized within the experiment were fungal neutral protease, fungal protease, fungal neutral protease and Liquipanol. The fungal acid protease was derived from *Aspergillus niger*, a pH optimum of 2.5-3.5, and a optimum temperature of 40- 55°C. Fungal protease was derived from *Aspergillus oryzae*, has a pH optimum of 3.0-6.0, and a temperature optimum of 50°C. Fungal neutral protease was derived from *Aspergillus oryzae*, a pH optimum of 5.0-7.0, and a temperature optimum of 37°C. Liquipanol was prepared from the dried latex of *Carica papaya L*, with a pH range of 5.0-7.0, and a temperature optimum of 40-70°C. The nine sorghum treatments included a control sorghum with no enzyme addition, fungal acid protease at 30 μL or 3μL/ 50-mL tube, fungal protease at 30 μL or 3μL/ 50-mL tube, fungal neutral protease at 30 μL or 3μL/ 50-mL tube, Liquipanol at 30 μL or 3μL/ 50-mL tube, all with sorghum grain as the substrate. Control treatments included sorghum and corn without added enzyme along with a blank. The enzymes were provided by Enzyme Development Corporation (Scranton, Pennsylvania).

On the day of the *in vitro* inoculation, enzyme solutions were mixed in a 1:1 v/v ratio with deionized water, transferred by pipette into centrifuge tubes, then placed into an orbital shaking incubator (New Brunswick Scientific, New Brunswick, New Jersey) set at a temperature of 39°C. Ruminal fluid was collected from a ruminally cannulated steer to be used as the inoculant. The cannulated steer was fed a diet consisting of 58.9% dry rolled corn, 30% wet corn gluten feed, 7% prairie hay, and a pelleted supplement. Ruminal fluid was strained through two layers of cheesecloth, deposited into pre-warmed insulated containers, and transferred to the Kansas State University Pre-Harvest Food Safety Laboratory (Manhattan, Kansas). The ruminal fluid was again strained through two layers of cheese cloth into separatory funnels and sparged with nitrogen gas then placed into an incubator for approximately 30 minutes to allow for stratification into layers. The bottom layer was discarded; the middle (microbial-rich) layer was used as the inoculant. A buffer solution [12] was mixed in a 4:1 ratio with the ruminal fluid inoculant.

To each centrifuge tube, 30 mL of buffered ruminal fluid were added, tubes were flushed with nitrogen, capped, and placed into the 39°C orbital shaking incubator at 25 rpm. The *in vitro* incubation lasted 36 hours for run 1 and 48 hours for run 2. After incubation tubes were removed from the incubator and placed on ice for 20 minutes to cease fermentation, and pH was measured (Orion Star A Series, Thermo-Fisher Scientific, Waltham, Massachusetts). A 1-mL fluid subsample was taken from the centrifuge tubes and mixed with 0.25 mL of 25% w/v m-phosphoric acid and frozen. Samples were then thawed, homogenized using a vortex mixer (Centrifuge 5415 C; Eppendorf, Hamburg, Germany), centrifuged at 14,000 rpm for 15 minutes. Supernatant (0.4 mL) was transferred by pipette to gas chromatography vials containing 1.2 mL of pivalic acid solution as internal standard. Concentrations of VFA were determined using an

Agilent 7890A GC system (Agilent Technologies; Santa Clara, California) equipped with a flame ionization detector and capillary column (DB-FATWAX UI 20 m x 0.180 mm x 0.18 μm film thickness; Agilent Technologies, Santa Clara, California). To determine *in vitro* dry matter digestibility (IVDMD), centrifuge tubes were placed into a freeze dryer (VirTis Genesis 35EL; ATS Scientific Products, Warminster, Pennsylvania) until completely dry, and weighed. The formula to calculate IVDMD is below.

$$IVDMD = \left[1 - \left[\frac{(Dried\ residue\ weight, g) - (Tube\ weight, g) - Blank}{Inital\ substrate\ weight\ g, (DM)}\right]\right]$$

$$Blank\ = Dried\ blank\ residue, g\ - Tube\ weight, g$$

Methane production and methane production per gram of substrate digested was determined by the adapted formulas below [13,14].

$$Methane\ yield, mM = \left[\frac{3.28 * (Acetate, mM + Butyrate, mM)}{Propionate, mM}\right] + 7.6$$

$$Methane\ production\ per\ gram\ of\ substrate\ digested = \frac{Methane\ yield, mM}{0.1 * \frac{IVDMD, \%}{1.5}}$$

3.2.2 Statistical Analyses

Statistical analyses were conducted as mixed models using treatment as the fixed effect and run and block within run as random effects, the (SAS 9.4; Cary, NC). The variables evaluated were VFA production, terminal pH, calculated methane production per gram of digested substrate, and

IVDMD. The least-squares means function was utilized to compare the variables to group similar treatments. Contrasts were performed to compare the average of the sorghum treatments compared to the control corn treatment, effect of enzyme addition, and effect of enzyme concentration.

3.3 Results and Discussion

Terminal pH of the sorghum-based treatments was 6.64 compared to the control corn treatment group at 6.46 ($P < 0.01$) indicating that more VFA accumulated within corn no enzyme treatment *in vitro* culture lowering terminal pH. There was no effect of enzyme addition ($P = 0.370$), there was a tendency for the high (30 μL) enzyme inclusion rate treatments to have greater a terminal pH than the low (3 μL) enzyme inclusion rate treatments ($P = 0.055$). Table 3-1 summarizes terminal pH of cultures with sorghum and corn-based treatments. Eun and Beauchemin (2005) [15] reported a decrease in mean ruminal pH when enzymes were supplemented to a low forage diet. While the pH of the sorghum based treatments within the present experiment was 6.64 when protease enzymes were added, the lower pH in the study by Eun and Beauchemin (2005) [15] could be attributed to barley being more degradable compared to that of sorghum grain [16]. Colombatto et al. (3003) [17] reported that addition of a protease enzyme to a continuous culture fermentation had no effect on pH. In contrast to results previously mentioned, Amaro et al. (2021) [18] reported a linear and quadratic decrease in pH when a neutral proteolytic enzyme and a linear and cubic decrease in pH when an alkaline proteolytic enzyme was added at increasing rates of 0, 0.25, 0.5, 0.75, and 1.0 mg/g of corn. Corn typically has greater digestibility than sorghum. The difference in the current study result and the results in previously reported [19] could be due to the decrease in starch and protein availability within sorghum grain [9] compared to digestibility values of corn. Terminal pH is correlated to digestibility; lower pH

indicates a greater digestibility. Additionally, greater fermentation leads to accumulation of organic acids in the cultures, which lowers the pH [20]. Results previously mentioned indicate that there were not increases in organic acid accumulation within the culture when exogeneous proteolytic enzymes were added to *in vitro* cultures. There was a tendency for terminal pH to be lower in the high inclusion rate treatments indicating that there were decreases in VFA accumulation within the culture.

In vitro dry matter disappearance is an assay that is utilized to estimate the digestibility of feedstuffs by anaerobic fermentation [21]. Results are depicted in Table 3.1. Dry matter disappearance was the greatest and pH lowest in the control corn treatment compared to the sorghum-based treatments ($P < 0.01$). No effects were observed for the effect of enzyme supplementation ($P = 0.228$), there was a decrease in IVDMD within the high (30 μL) inclusion rate treatments compared to the low (3 μL) inclusion rate ($P < 0.01$). These results indicate that there was no difference in IVDMD between the sorghum no enzyme treatment and the average of the enzyme addition treatments, however, when the enzymes were included in the culture at the high (30 μL) there was an inhibitory effect on IVDMD. Within the enzyme treatments there was a tendency for fungal neutral protease to decrease dry matter digestibility compared to the control sorghum treatment ($P = 0.092$). This result could be due to the temperature optimum of the enzyme being slightly lower than what the in vitro fermentation was held at. In contrast to the results being reported in the present study, Vera et al. (2012) and Eun and Beauchemin (2007) [22,23] reported an increase in dry matter (DM) degradability when diets were treated a proteolytic enzyme. Colombatto et al. (2003) [17] increase in IVDMD when a neutral or alkaline proteolytic enzymes were added at increasing rates 0, 0.25, 0.5, 0.75, and 1.0 mg/g of corn. Results for IVDMD in the current experiment validate the pH results mentioned previously. The

treatment with the lowest recorded pH also had the greatest IVDMD. Culture pH and IVDMD were similar across the treatments with enzyme addition ($P > 0.10$).

Acetate is the primary VFA produced within the rumen, followed by propionate, then butyrate. There was no effect of the enzyme addition or enzyme inclusion rates to sorghum in the liquipanol, fungal acid protease, fungal neutral protease, or fungal protease treatment ($P > 0.10$). Indicating that enzyme supplementation along with the rate of enzyme addition had no effect on acetate production. Similar to the results previous reported by Eun and Beauchemin (2005) and Colombatto et al. (2003) [15,17] no difference in acetate production were observed in previous response to proteolytic enzyme supplementation. In contrast, Vera et al. (2012) [22] reported a decrease in acetate production when TMR was supplemented with proteolytic enzymes. When evaluating a neutral and alkaline proteolytic enzyme at three supplementation rates, no differences were observed among treatments [18]. Propionate is the second most abundant VFA produced within the rumen through anaerobic fermentation. There was no effect of enzyme addition for liquipanol, fungal neutral protease, or fungal acid protease treatments ($P > 0.10$). When fungal protease was added at the high and low inclusion rates, propionate production was decreased compared to the control sorghum treatment ($P = 0.030$). Propionate production was decreased in the high (30 μL) enzyme inclusion rate compared to the low (3 μL) inclusion rate ($P < 0.10$). Similarly, Eun and Beauchemin (2005) and Vera et al. (2012) [15,22] reported no effect of protease addition on propionate production. Amaro et al. (2021) [18] reported no effect on propionate production when alkaline or neutral proteases were supplemented at 0, 0.25, and 0.50 mg/g of corn. Butyrate is the third most abundant VFA within the rumen, no enzyme supplementation had an impact on butyrate concentration ($P < 0.10$). Sutton et al. (2003) [24] reported that butyrate production was reduced when pH within the rumen was reduced. This is

validated by the results reported within the present study. Terminal pH and butyrate production were correlated, as pH decreased butyrate production decreased. Eun and Beauchemin (2005) and Vera et al. (2012) [15,22] reported similar results, observing that enzyme addition did not influence butyrate production. Amaro et al. (2021) [18] reported no difference in butyrate production when a neutral protease was supplemented at 0, 0.25, and 0.75 mg/g of corn. When an alkaline protease was supplemented at the 0, 0.25, and 0.75 there was a tendency for butyrate production to decrease quadratically. Isovalerate production is a branched-chain volatile fatty acid (BCVFA) that is a byproduct within the rumen from degradation of protein within the rumen [2]. Isovalerate was produced in greater amounts in the control corn treatment when compared to the sorghum-based treatments ($P = 0.029$). There was a tendency for greater amounts of isovalerate to be produced when enzymes were included in the sorghum-based treatments ($P = 0.087$). Isovalerate production was increased when proteolytic enzymes were supplemented at the high (30 μL) inclusion rate ($P < 0.10$) From these observations we can conclude that supplementation of proteolytic enzymes increase degradation of leucine within the sorghum based treatments [25]. In contrast, Vera et al. (2012) [22] reported no difference in isovalerate production when a protease enzyme was included within the continuous culture. Colombatto et al. (2003) [17] reported no difference in isovalerate production when alkaline or neutral proteases were supplemented at 0, 0.25, and 0.50 mg/g of corn. Valerate is a VFA that is metabolized from proline within the rumen [26]. These results indicate that there was no effect of the proteolytic enzymes on the breakdown of proline within the rumen. Similarly, Eun and Beauchemin (2005) and Vera et al. (2012) [15,22] reported no effect of alkaline enzyme supplementation on valerate production. The results presented in the current experiment for valerate and isovalerate align with sorghum grain containing greater levels of leucine compared

to proline. Values differed amongst varies of sorghum grain, but concentrations of leucine were consistently greater than that of the values within corn [27]. Total VFA production in the control corn treatment was greater higher than in the sorghum-based treatments ($P < 0.01$). No effect of enzyme addition to sorghum was observed for total VFA production ($P = 0.642$). The higher levels of VFA within the rumen, the pH decreases [20]. Given that there were minimal differences among the individual VFAs, there was no expected difference for total VFA production. Similarly, Amaro et al. (2021) [18] reported no difference in total VFA production when an alkaline or neutral protease were added to the in vitro culture at a rate of 0, 0.25, and 0.75 mg/g of corn. In contrast, Vera et al. (2012) [22] reported an increase in total VFA production when proteolytic enzymes were added to barley based diets with or without DDGS. The increase in total VFA production could be due to barley being more rapidly fermentable compared to sorghum [16]. The acetate to propionate ratio (A:P) is a beneficial measure of efficiency within the rumen. The greatest A:P ratio observed was from the control corn treatment ($P < 0.01$). When Liquipanol, fungal protease, and fungal neutral proteases were included in the *in vitro* culture with sorghum the A:P ratio increased as inclusion level increased compared to the control sorghum treatment ($P < 0.05$). Fungal acid protease tended to have an increase the A:P when added to the in vitro culture ($P = 0.056$). Reducing the (A:P) within the rumen can lead to improved ruminal efficiency [28]. Decreased A:P have greater rates of propionate production, which utilizes H_2 within the rumen, suppressing methanogenesis within the rumen. When there is less methane produced within the rumen there tends to be less energy wasted [29]. The increase in A:P indicates that when the proteolytic enzymes were added to the *in vitro* cultures with sorghum gain there is a less energetically efficient fermentation process. In contrast, Amaro et al. (2021) [18] reported a tendency for the rumen to be more energetically

efficient when supplemented a fungal neutral protease to corn. Corn being more readily digestible compared to sorghum could account for the contrasting in results from Amaro et al. (2021) compared to the results reported in the current experiment. Eun and Beauchemin (2005) and Vera et al. (2012) [15,22] reported no difference in A:P when an alkaline proteolytic enzyme was added to the TMR.

Methane production was calculated using the equation: MY = 3.28 × (acetate + butyrate)/propionate + 7.6 [14]. Methane production per gram of substrate digested was then calculated using the value from the previous equation in the equation: Methane production per gram of substrate digested = methane yield / (0.1* (IVDMD/1.5)). Within the rumen free H_2 produced from other processes are used to produce methane through methanogenesis. Methanogenesis is an inefficient process within the rumen. When H_2 production or availability is decreased within the rumen methane production is decreased [30]. Diverting H_2 away from methanogenesis has the potential to decrease methane emissions, while increasing propionate production, and in turn making the animal more efficient [31] Methane production can represent up to 12% of gross energy lost [32]. Methane production per gram of substrate digested was the lowest in the corn treatment ($P < 0.01$). Fungal neutral protease increased the amount of methane produced per gram of substrate digested when added at a rate of 30 or 3 μL. There was a tendency for increase in methane produced per gram of substrate digested when proteolytic enzymes were added to the *in vitro* cultures. Soltan et al. (2021) [33] reported differing results in an experiment utilizing growing lambs. The authors reported no difference in methane production per kg of digestible organic matter between the corn and sorghum. In the present study, there was a tendency for methane/g to be greater in the enzyme treated sorghum-based

treatments. Unlike the current study, no differences were observed in methane production between treatments when protease enzymes were added [17,22].

3.5 Conclusion

Results from this experiment revealed small but non-significant improvement in *in vitro* dry matter disappearance and VFA production with addition of exogeneous protease enzymes.

Conflict of Interest

The authors declare no conflict of interest

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Author Contributions

Conceptualization, J.D.; methodology, J.D.; validation, J.D. and E.K.; formal analysis, J.D.; investigation, J.D. and E.K.; data curation, J.D. and E.K.; writing—original draft preparation, E.K.; writing—review and editing, J.D. and L.M.; visualization, J.D.; supervision, J.D.; project administration, J.D.; funding acquisition J.D.

Data Availability Statement

The raw data supporting conclusions of this article will be available from the authors upon request.

Institutional Review Board Statement

Ethics Approval – Procedures for this experiment were approved by the Kansas State University Institutional Animal Care and Use Committee, license number 4533.

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Table 3-1: Effect of protease enzyme addition to 0.3 g and 0.5g of substrate at differing inclusion rates on pH and IVDMD.

Treatment[1]	Terminal culture pH	IVDMD[4], %
Corn	6.47[c]	75.77[a]
Sorghum	6.63[ab]	68.39[bc]
Fungal acid protease low[3]	6.63[ab]	68.20[bc]
Fungal acid protease high[3]	6.66[ab]	65.63[bc]
Fungal protease low[3]	6.68[ab]	69.39[b]
Fungal protease high[3]	6.69[a]	65.39[cd]
Fungal neutral protease low[3]	6.60[b]	68.09[bc]
Fungal neutral protease high[3]	6.66[ab]	64.07[d]
Liquipanol low[3]	6.63[ab]	67.73[bc]
Liquipanol high[3]	6.67[ab]	67.34[bc]
SEM[2]	0.029	1.405
P-value		
Treatment effect	<0.01	<0.01
Effect of Liquipanol	0.473	0.517
Effect of fungal acid protease	0.557	0.275
Effect of fungal protease	0.107	0.453
Effect of fungal neutral protease	0.851	0.092
Corn vs average of sorghum treatments[5]	<0.01	<0.01
Effect of enzyme addition on sorghum[6]	0.370	0.228
Effect of enzyme concentration[7]	0.055	0.001

[*]Numbers with like superscripts do not differ, P>0.05.

[1]Low enzyme inclusion rate = 3µL per 50 mL centrifuge tube; High enzyme inclusion rate = 30µL per 50 mL centrifuge tube.

[2]Standard error of the mean.

[3]Substrate utilized was sorghum.

[4]IVDMD = *In vitro* dry matter digestibility, to determine *in vitro* dry matter digestibility (IVDMD), centrifuge tubes were placed into a freeze dryer (VirTis Genesis 35EL; ATS Scientific Products, Warminster, Pennsylvania) until completely dry, and weighed. The formula to calculate IVDMD is below.

$$IVDMD = \left[1 - \left[\frac{(Dried\ residue\ weight, g) - (Pan\ weight, g) - Blank}{Inital\ substrate\ weight\ g, (DM)}\right]\right]$$

[5]Compares the corn no enzyme treatment to the average of all 9 sorghum-based treatments.

[6]Compares the sorghum no enzyme treatment compared to the average of the 8 enzyme addition treatments.

[7]Compares the low and high enzyme addition rates.

Table 3-2: Effect of protease enzyme addition at to 0.3g and 0.5g of substrate differing inclusion rates on VFA and methane production.

Treatment*	A[4]	P[4]	I[4]	B[4]	IV[4]	V[4]	VFA[4]	A:P	CH_4/g[5]
Corn	63.69[a]	37.11[a]	1.12[a]	15.78[c]	3.61[a]	2.41[a]	123.71[a]	1.73[a]	29.20[e]
Sorghum	52.33[c]	33.94[b]	0.71[bc]	18.81[ab]	3.34[cd]	2.25[abc]	111.37[bc]	1.53[de]	31.83[d]
Fungal acid protease low[3]	52.66[bc]	34.36[bc]	0.65[c]	17.50[b]	3.22[d]	2.09[c]	110.54[bc]	1.54[e]	31.55[d]
Fungal acid protease high[3]	52.78[bc]	32.66[cde]	0.85[b]	17.65[ab]	3.51[abc]	2.16[bc]	109.61[c]	1.62[b]	33.75[abc]
Fungal protease low[3]	52.85[bc]	33.56[bc]	0.79[bc]	18.53[ab]	3.43[abc]	2.32[ab]	111.48[bc]	1.59[cd]	31.65[d]
Fungal protease high[3]	52.95[bc]	32.30[e]	0.80[bc]	17.67[ab]	3.53[ab]	2.16[bc]	109.40[c]	1.65[b]	34.08[ab]
Fungal neutral protease low[3]	53.47[bc]	34.14[b]	0.79[bc]	19.01[a]	3.47[abc]	2.32[ab]	113.20[b]	1.58[cd]	32.36[cd]
Fungal neutral protease high[3]	53.17[bc]	32.47[de]	0.79[bc]	17.66[ab]	3.58[a]	2.16[bc]	109.82[c]	1.64[b]	34.77[a]
Liquipanol low[3]	52.17[c]	33.57[bc]	0.84[b]	18.78[ab]	3.39[bcd]	2.27[ab]	111.02[bc]	1.56[de]	32.32[cd]
Liquipanol high[3]	53.85[b]	33.43[bcd]	0.89[b]	18.04[ab]	3.59[a]	2.20[bc]	111.99[bc]	1.61[bc]	32.81[bcd]
SEM	0.545	0.413	0.076	0.644	0.106	0.086	1.276	0.016	0.644
P-value									
Treatment effect	<0.01	<0.01	<0.01	0.001	0.001	0.012	<0.01	<0.01	<0.01
Effect of Liquipanol	0.258	0.345	0.051	0.056	0.082	0.837	0.913	0.017	0.334
Effect of fungal acid protease	0.512	0.355	0.616	0.462	0.751	0.102	0.286	0.056	0.253
Effect of fungal protease	0.345	0.030	0.262	0.271	0.092	0.865	0.445	<0.01	0.149
Effect of fungal neutral protease	0.101	0.168	0.306	0.462	0.029	0.869	0.911	<0.01	0.016
Corn vs sorghum treatments[6]	<0.01	<0.01	<0.01	<0.01	0.029	0.004	<0.01	<0.01	<0.01
Effect of enzyme addition[7]	0.207	0.118	0.184	0.208	0.088	0.528	0.642	<0.01	0.085
Effect of enzyme concentration[8]	0.253	<0.01	0.160	0.059	<0.01	0.067	0.055	<0.01	<0.01

*Low enzyme inclusion rate = 3µL per 50-mL centrifuge tube; High enzyme inclusion rate = 30µL per 50-mL centrifuge tube.

[1]Abbreviations, A = Acetate, mM; P = Propionate, mM; I = Isobutyrate, mM; B = Butyrate, mM; V = Valerate, mM; IV = Isovalerate, mM; VFA = Total VFA, mM; A:P = Acetate:Propionate ratio; CH_4/g = Methane per gram of substrate digested, millimoles/g.

[2] Numbers with like superscripts do not differ, P>0.05.

[3]Substrate utilized was sorghum.

[4]Liquid for VFA analysis was centrifuged at 14,000 rpm (Centrifuge 5415 C; Eppendorf, Hamburg, Germany). Once centrifuged 0.4 mL of supernatant was transferred to chromatography vials, 1.2 mL of pivalic acid was added to the vials to act as the internal standard. Concentrations of VFA were determined by using an Agilent 7890A GC system (Agilent Technologies; Santa Clara, California) equipped with a flame ionization detector and capillary column (DB-FATWAX UI 20 m x 0.180 mm x 0.18 µm film thickness; Agilent Technologies, Santa Clara, California).

[5]Methane production per gram of substrate digested was determined by the formula Patra et al. (2016) and Williams et al. (2019).

$$Methane\ production\ per\ gram\ of\ substrate\ digested = \frac{Methane}{0.1 * \frac{IVDMD}{1.5}}$$

[6]Compares the corn no enzyme treatment to the average of all 9 sorghum-based treatments.

[7]Compares the sorghum no enzyme treatment compared to the average of the 8 enzyme addition treatments.

[8]Compares the low and high enzyme addition rates.