THESIS

EFFECTS OF *ASPERGILLUS ORYZAE* α-AMYLASE SUPPLEMENTATION ON RUMEN VOLATILE FATTY ACID PROFILE AND RELATIVE ABUNDANCE OF MRNA ASSOCIATED WITH NUTRIENT TRANSPORTERS IN RUMINAL AND DUODENAL TISSUE ON BEEF STEERS

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ABSTRACT

EFFECTS OF *ASPERGILLUS ORYZAE* α-AMYLASE SUPPLEMENTATION ON RUMEN VOLATILE FATTY ACID PROFILE AND RELATIVE ABUNDANCE OF MRNA ASSOCIATED WITH NUTRIENT TRANSPORTERS IN RUMINAL AND DUODENAL TISSUE ON BEEF STEERS.

The objective of this study was to investigate the impact of *Aspergillus oryzae* α-amylase (AAM) supplementation on rumen volatile fatty acid (VFA) profile and relative abundance of mRNA associated with nutrient absorption in ruminal and duodenal tissue from beef steers. Nine crossbred beef steers (BW 622 ± 50 kg), fitted with rumen and duodenal fistulas were used in this experiment. Steers were housed in individual stations and fed a high concentrate finishing diet (74.6% corn on a DM basis) twice daily for 8 d. Treatments included 1) CON (5 g corn meal; n=5) and 2) AAM (5g 750 fungal α-amylase units/g; n=4). Dietary treatment supplements were manufactured prior to each feeding by mixing 3 g of α-amylase or corn meal into 150 g of dried distiller's grains (DDG) for the AM feeding and 2 grams of α-amylase or corn meal into 100 g of DDG for the PM feeding. Supplements were applied as a top dress for every feeding and thoroughly mixed by hand. On d 5, rumen fluid samples were obtained every 4 h for 24 h and analyzed for VFA. On d 9, rumen papillae and duodenal mucosal tissue samples were collected. Total tissue RNA was extracted for real-time PCR analysis. Sodium/potassium ATPase pump α1, glucose transporter 2 and 5, putative anion transporter, isoform1, sodium/hydrogen antiporter isoforms1, 2 and 3, 3-hydroxy 3-methylglutaryl coenzyme A synthase isoform2, down regulated in adenoma, monocarboxylate co-transporter isoform1, and

glyceraldehyde-3-phosphate dehydrogenase mRNA were tested. Relative expression (fold change) of mRNA in ruminal and duodenal tissues were analyzed using PROC GLM and VFA distribution was analyzed using PROC MIXED as a randomized block design with repeated measures. No treatment differences were detected for any of the genes analyzed in ruminal or duodenal tissue. Concentrations of VFA and the acetate to propionate ratio were similar across treatments. However, the acetate:propionate ratio and molar percentages of butyrate were numerically greater in AAM steers compared to controls. Under the conditions of this experiment, AAM supplementation had no impact on relative expression (fold change) of mRNA associated with nutrient absorption and minimal impacts on molar proportions of VFA.

KEY WORDS

Duodenum, fungal α-amylase, gene, rumen, steer, and volatile fatty acids

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TABLE OF CONTENTS

ABSTRACT ii
KEY WORDS iv
ACKOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES viii
LIST OF FIGURES ix

CHAPTER I: Literature Review 1
INTRODUCTION 1
BACKGROUND 2
Gastrointestinal tract anatomy 2
Rumen epithelium 3
Nutrient digestion and absorption in ruminants 4
Rumen pH 6
Short chain fatty acids 7
Acidosis 8
Laminitis 9
Bloat 9
Direct- fed Microbial *Aspergillus oryzae* 10
Nutrigenomics 11
SUMMARY 12
LITERATURE CITED 13

CHAPTER II: Effects of *Aspergillus oryzae* α-amylase supplementation on rumen volatile fatty acid profile and relative abundance of mRNA associated with nutrient transporters in ruminal and duodenal tissue 16
SUMMARY 16
INTRODUCTION 17
MATERIALS AND METHODS 18
Animal Care 18
Ruminal Papillae and Duodenal Mucosal Collection 19
Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis 20
Semi-quantitative real-time PCR 20

Ruminal Volatile Fatty Acids Collection....21
Statistical analysis....21
RESULTS AND DISCUSSION....22
Ruminal Volatile Fatty Acids....22
Gene expression....22
LITERATURE CITED....31

APPENDIX I....33
BATCH SHEETS....34

APPENDIX II....35
SAS PROC PRINT....36
Relative expression (fold change) of mRNA in rumen tissue samples data....36
Relative expression (fold change) of mRNA in duodenum mucosal tissue samples data....37
Volatile fatty acid distribution data....38

APPENDIX III....39
SAS DATA....40
Relative expression (fold change) of mRNA in rumen tissue samples data....40
Relative expression (fold change) of mRNA in duodenum mucosal tissue samples....41
Volatile fatty acid distribution data....42

APPENIX IV....44
SAS CODE....45
Analyze relative expression (fold change) of mRNA in rumen tissue samples data....45
Analyze relative expression (fold change) of mRNA in duodenum mucosal tissue samples data....48
Analyze volatile fatty acid distribution data....51

LIST OF TABLES

Table 1. Dry matter composition of diets for the steers supplemented with *Aspergillus oryzae* α-amylase. 26

Table 2. Primer and probe sequences and National Center for Biotechnology Information accession numbers for quantitative real-time PCR analysis. 27

Table 3. Molar percentage of ruminal Volatile Fatty Acid concentrations of the steers supplemented with A. oryzae α-amylase. 28

Table 4. Relative expression (fold change) of mRNA in the rumen of steers supplemented with A. oryzae α-amylase. 29

Table 5. Relative expression (fold change) of mRNA in the duodenum of steers supplemented with A. oryzae α-amylase. 30

Table 6. Batch sheets for the steers supplemented with *Aspergillus oryzae* α-amylase. ..34

LIST OF FIGURES

Figure 1. Drawing from an electron micrograph of ruminal epothelium layers. Stratum corneum. 4

Figure 2. Pathways of carbohydrate metabolism in rumen. 5

Figure 3. Proteins participation in maintenance of the rumen intracellular pH and their putative functions. 7

Figure 4. Partial model depicting the current understanding for SCFA absorption inrelation to the stabilization of ruminal pH. 8

CHAPTER I: Literature Review

INTRODUCTION

The growing population, decreasing rural labor force, diminishing viable farm land and unpredictable climate change has increased the demand of producing food in the most efficient manner. With the improvements associated with producing cattle and beef, some negative effects have occurred for the consumer, animal, and environment. It has become a battle to eliminate these negative issues without decreasing the efficiency of production. The following review will focus on ruminant anatomy and function, nutrient digestion, metabolic disorders, and direct fed-microbial feed additives.

The price of grains to relative to forages has caused a shift in cattle production from systems that were dependent upon forages to systems dependent upon grains. Some problems have occurred from converting cattle from high roughage diets to high concentrate diets. Starch from cereal grains is the primary dietary energy source for finishing cattle, representing 50 to 70% of the finishing diet. Amylose and amylopectin comprise most of the starch in the finishing beef cattle diet (Huntington, 1997). The rate and extent of starch digestion in the rumen has an impact on total tract starch digestibility and ruminant performance (Lykos et al., 1997). Extensive ruminal starch digestion is beneficial; however, rapid starch fermentation could possibly result in acidosis (Owens et al., 1998). Understanding the correct balance for optimizing ruminal starch fermentation, while avoiding conditions leading to ruminal upset is desirable for preventing acidosis in beef cattle.

Some producers have used ionophores to help with preventing acidosis. Ionophores will prevent acidosis in animals that diets have rapidly changed from a roughage diet to high

carbohydrate diet (Oehme and Pickrell, 1999). Ionophores are antibiotics that manipulate the composition of the microflora in the rumen and improve feed efficiency and rate of gain in cattle. The ionophores reduce protein degradation in the rumen which aids in post ruminal digestion (Horton et al., 1992). However, with the increasing demand and development of more natural markets for beef, a natural substitute for feed grade antibiotics and ionophores needs to be explored. Direct-fed microbials (DFM) could possibly be the alternative. Direct-fed microbials or probiotics are defined as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" (Heyman and Menard, 2002).

BACKGROUND

Gastrointestinal tract anatomy

Ruminants have unique digestive system that allows the animal to utilize energy from a wide variety of feed sources through fermentation. The anatomy of the digestive system includes the mouth, tongue, salivary glands, esophagus, four compartment stomach (rumen, reticulum, omasum, abomasum), pancreas, gall bladder, small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). The ruminant stomach occupies almost 75 percent of the abdominal cavity.

The first compartment of the stomach is the rumen. Within the rumen the digesta is not uniform, especially when ruminants are fed a high fiber diet, and is separated into three levels. The top level is the gas which sits on the middle level consisting of the fiber mat. The bottom level consists of liquid. The rumen is lined with papillae and divided into dorsal, ventral, caudodorsal, and caudoventral sacs. The rumen's main role is microbial fermentation and stratification and mixing of digesta to aid in further digestion in the alimentary canal. The pH is typically 6.5 to 6.8 when roughage is fed; however, pH can be much lower with a high grain diet.

The rumen can hold up to 190 liters of digested feed. This feed is digested by bacteria, protozoa, and fungi within the rumen. The microbes in the rumen are able to digest fiber and produce microbial protein and volatile fatty acids (VFA). Volatile fatty acids are one of the main by-products of rumen fermentation and are absorbed through the rumen wall. Rumen development is critical to ensure proper digestion and nutrient absorption of a ruminant's diet. Obtaining a better understanding of how exactly the rumen functions will not only help ruminants' well-being but production efficiency as well.

The second compartment of the stomach is the reticulum. The reticulum is known for its honeycomb wall structure, and can hold approximately 19 litters. This honeycomb wall structure aids in trapping large feed particles to digest it further before being regurgitated, re-chewed, and re-swallowed to be further digested.

Following the reticulum is the omasum. Its main function is to filter. The folded wall structure of the omasum aids in filtering the feed particles and squeezing the majority of the water out. The last compartment of the stomach is the abomasum. The abomasum is the "true stomach". It has a low pH from HCl secretion and excretes enzymes to initiate protein digestion.

Rumen epithelium

The rumen is lined with stratified squamous epithelium covered with papilla which allows absorption of VFA (Sehested et al., 1999, 1996). Propionic and butyric acid stimulate papillary growth (Sander et al., 1959). However, butyric acid is far more effective than propionic acid and appears to be the most effective VFA in stimulating the development of the rumen epithelium (Sander et al., 1959). Initially for young calves, the papillae are nonfunctional, but grow larger and start functioning as calves mature. There are four cell layers of the rumen epithelium. Starting from the luminal side the first layer is stratum corneum, stratum

granulosum, stratum spinosum, and stratum basale which are shown in Figure 1. The linings of the cells have an abundance of mitochondria and allow active transport of nutrients. The thicknesses of the epithelium and the diet have potential effect on the transport of nutrients (Weigand et al., 1972).

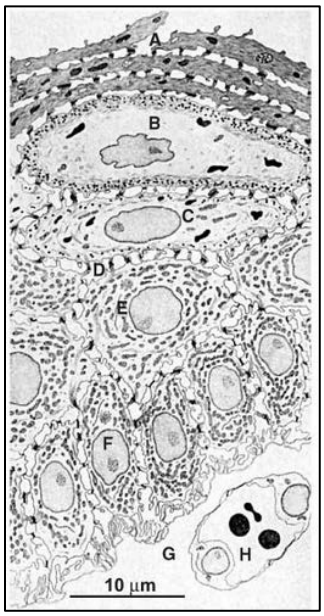

Figure 1. Drawing from an electron micrograph of ruminal epithelium layers. Stratum corneum (A), swollen cells of stratum granulosum (B), flat cells of the stratum granulosum (C), intercellular canaliculi (D), stratum spinosum with desmosomes (E),and stratum basale (F). (Eurell and Frappier, 2013)

Nutrient digestion and absorption in ruminants

When ruminants ingest food, the nutrients which supply energy are initially in the form of carbohydrates, proteins, and lipids. These three products are digested and converted into suitable products that can be used directly by the animal or microbes in the rumen. Initial breakdown of feed particles, start with the mechanical forces of chewing. The saliva of ruminants has no amylase so there is little breakdown of carbohydrate by saliva. Saliva contains salivary lipase that aids in digestion of lipids, but saliva's main role is to lubricate the feed bolus and to buffer pH in the reticulum and rumen. Chemical action and enzymatic activity occurring

in the abomasum also aids in the digestion of feed particles. Microbial fermentation occurs in the rumen, cecum, and colon. Most ruminants break down the majority of carbohydrates (e.g. starch and cellulose) into simple sugars through fermentation in the rumen by microbes. The microbes use these simple sugars as energy for growth. Only 5 to 20% of consumed carbohydrates are digested in the small intestine (Huntington, 1997). In the process of carbohydrate fermentation, microbes produce acetate, propionate, butyrate, and the by-products carbon dioxideand methane. Figure 2 shows the possible pathways of carbohydrate metabolism in the rumen.

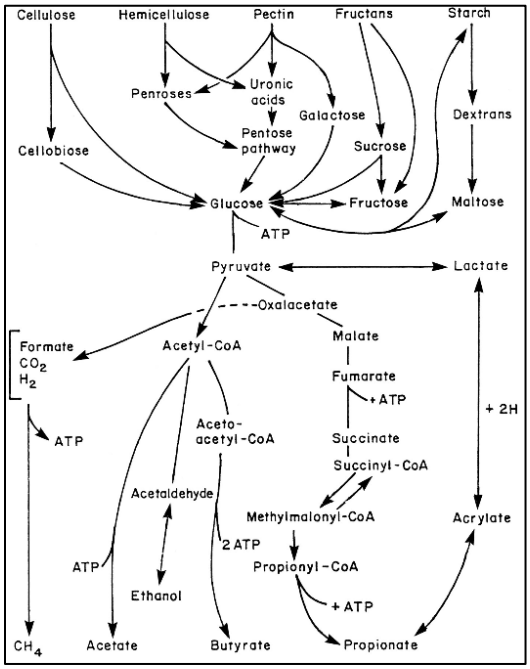

Figure 2. Pathways of carbohydrate metabolism in rumen. (Van Soest, 1994)

Protein can come either from the diet or from microbes in the rumen. Microbes have requirements for protein and energy to facilitate growth and reproduction. However, through digestion and contractions some of the microbes get carried to the abomasum where they are digested as protein. When protein is digested, it is broken down into poly-peptides which are

broken down further into amino acids and then eventually ammonia. Excess ammonia is absorbed through the rumen wall. Once through the rumen wall it is converted into urea in the liver and returns through the blood and incorporated into saliva or is excreted by the body.

Lipids are a source of energy for the animal. During microbial fermentation of lipids, some vitamins required by the animal are also produced. Lipid digestion results in the release of glycerol from triglycerides, which along with phospholipids, are fermented into VFA. Unsaturated fatty acids are hydrogenated in the rumen to mostly saturated fatty acids before passing through the abomasum and being absorbed from the small intestine.

Rumen pH

Sustaining an ideal rumen pH is critical for ruminants. It also contributes to VFA absorption and the growth and metabolism of rumen microbes (Van Soest, 1994). Effective rumen pH control is critical to maintaining a healthy rumen. All diets produce acidic products in the rumen but bicarbonate from saliva buffers the rumen fluid and stabilizes the rumen pH. When high grain diets are fed in an attempt to maximize the limits of cattle growth, saliva alone cannot maintain an ideal pH. Maintenance of rumen intracellular pH is accomplished partly through exchange proteins, such as sodium/hydrogen exchange (NHE) proteins and monocarboxylate transporter isoform 1 (MCT1) that regulate cellular H^+ efflux (Gäbel and Aschenbach, 2006) and transport VFA metabolites into the blood (Muller et al., 2000). This process is illustrated in Figure 3.

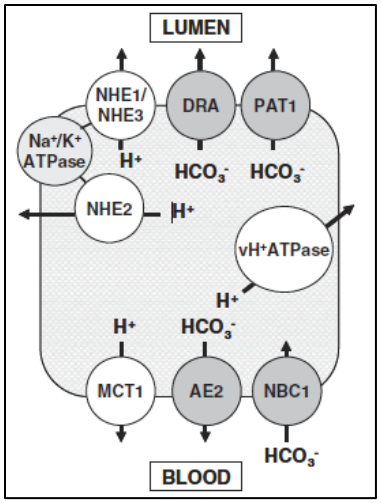

Figure 3. Proteins participation in maintenance of the rumen intracellular pH and their putative functions. NHE1: Na+/H+ exchange protein 1; NHE2: Na+/H+ exchange protein 2; NHE3: Na+/H+ exchange protein 3; DRA: down regulated in adenoma; PAT1: putative anion transporter; AE2: anion exchanger 2; H^+: proton; HCO_3^-: bicarbonate ion; NBC1: Na^+/HCO_3- cotransporter 1; H^+ ATPase: vacuolar-type ATPase. (Connor et al., 2010).

Short chain fatty acids

During ruminal fermentation a large amount of VFA are produced. The main three acids produced are acetate, propionate, and butyrate. They are all absorbed through the rumen epithelium and transported to the blood. This is possible by passive diffusion, one proton is removed from the rumen contents and as the VFA enter and dissociate in the cytosol. The proton that was removed needs to be ejected to maintain intracellular pH. Sodium/hydrogen exchangers (NHE) can carry these protons back to the lumen or into extra-cellular spaces. This is illustrated in Figure 4. It is important for this diffusion to occur to help prevent excessive and damaging drops in rumen pH. Acetic and propionic acid travel to the liver. Acetic acid leaves the liver and is oxidized throughout the body to produce ATP or acetate maybe used to synthesize lipids. Propionic acid in the liver acts as a major substrate for gluconeogenesis thus producing glucose that may be used by all tissues. Butyrate is partly metabolized by the rumen epithelium to a ketone body, beta-hydroxybutyrate, which is used for energy production by different tissues. If

these acids are not absorbed by the epithelium at a normal rate their dissociation in the ruminal fluid can lead to a reduction in ruminal pH and the onset of acidosis. A sudden change in diet can cause an increase of VFA and lactic acid in the rumen, which can lead to a digestive disorder called acidosis.

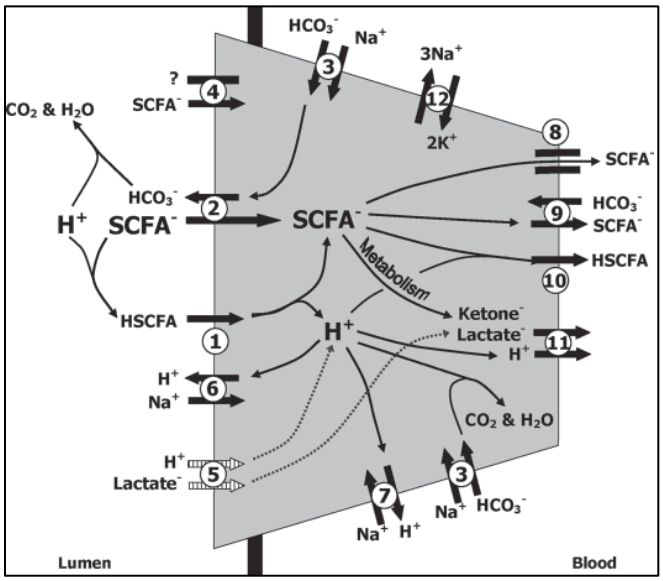

Figure 4. Partial model depicting the current understanding for SCFA absorption in relation to the stabilization of ruminal pH. (1) Diffusional absorption of SCFA facilitates the removal of a proton associated with the SCFA. This proton will rapidly dissociate in the cytosol where it can be exported by sodium/hydrogen exchanges (6, 7) or coupled with metabolites of SCFA (e.g. ketone bodies and lactate) via the monocarboxylate transporter (3) or a basolateral ion channel (9). Dissociated SCFA can be absorbed in an anion exchange mechanism thereby providing a source of bicarbonate to the ruminal contents (2). This bicarbonate can then neutralize a proton through the carbonic anhydrase reaction. The bicarbonate supply to the epithelia is derived from blood (4, 5). (Aschenbach et al., 2011).

Acidosis

There are two types of acidosis that can occur, acute and sub-acute. Acute acidosis usually occurs when the rumen pH suddenly drops to 5.2 or less. Most commonly acute acidosis follows a change in diet that is high in rapidly fermentable carbohydrates. Clinical signs of acute acidosis include complete anorexia, abdominal pain, rapid beating of the heart, abnormally fast

breathing, diarrhea, lethargy, staggering, decumbency and death (Krause and Oetzel, 2006). Slowly reducing fiber and increasing concentrate into diets, adding roughage to dilute the starch in diets, or supplementing buffers into the diet helps prevent this disorder.

Sub-acute ruminal acidosis, also known as SARA, is similar to acute acidosis, but is when the rumen pH stays below 5.2 for a long period time. Lactic acid however, does not constantly accumulate in the rumen fluid of cattle affected with SARA (Oetzel, 1999). Beauchemin and McAllister state that cattle with sub-acute acidosis can experience diarrhea, weight loss, reduced milk production, and increased susceptibility to other metabolic disorders. Acidosis may also induce other health issues such as lameness and bloat.

Laminitis

The onset of laminitis in dairy cattle may be influenced by the occurrence of sub-acute rumen acidosis (Cook et al., 2004). Lameness in cattle is a major health and welfare concern especially in dairies. Laminitis is a general term that refers to inflammation of the connective tissue in the hoof (Krause and Oetzel, 2006). The link between the two health issues relates back to the pH change in the rumen. As the pH drops it signals a vasoactive mechanism that increases the blood flow to cattle's hooves (Nocek, 1997). If laminitis is not caught early on, it can cause major production and animal welfare problems. Trimming of the hooves can help the animal's wellbeing, but can cause stress on the animal while doing so and cost the producer time and money. The best approach is prevention for both the producer and animal.

Bloat

Bloat in cattle is a main concern for feedlot productions. Bloat is a digestive disorder that occurs when the eructation mechanism is inhibited and the production of gas from the rumen exceeds the animal's ability to expel the excess gas. This gas formed by microbes in the rumen

is normal and occurs during fermentation. Extracellular bacterial mucopolysaccharides and stored carbohydrates released during microbial cell lysis increases the viscosity of rumen fluid resulting in the trapping of the gas, and forming the stable foam that leads to bloat (Cheng et al., 1998).

To find the correct balance for optimizing ruminal starch fermentation while avoiding conditions leading to ruminal upset is desirable for preventing acidosis and other related metabolic disease that occur from acidosis in beef cattle. To prevent the occurrence of these diseases while maintaining the same diet different supplements have been added. With the increasing demand and development of more natural markets, a natural substitute for antibiotics and ionophores has been created.

Direct- fed Microbial *Aspergillus oryzae*

Direct- fed Microbials (DFM) or probiotics are defined as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" (Heyman and Menard, 2002). Direct fed microbials may increase daily gain, feed efficiency, improve health and ruminal acidosis (Ghorbani et al., 2002). Direct-fed microbials for ruminants can be from fungi or bacteria cultures. *Aspergillus oryzae* is a fungus, produces α-amylase that aids in the digestion of starch. *Aspergillus oryzae* a filamentous fungus and is used in Chinese, Koran, and Japanese cuisine to ferment soybeans. Tricarico et al. (2005) suggested that supplemental fungal α-amylase did not increase ruminal starch digestion but consistently increased butyrate and reduced propionate molar proportions in the rumen, indicating that microbial starch digestion profile may have been altered but not total ruminal starch digestibility. Increasing butyrate could potentially help with rumen development. Improving rumen

development would help with the absorption of nutrients and VFA which may increase feed efficiency and improve the rumen environment.

Nutrigenomics

Nutrigenomics attempts to study the genome-wide influences of nutrition. Genome sequencing information has allowed physiological research to aid in the understanding of rumen development, nutrient uptake, and production efficiency of ruminants. Nutrigenomics' goal is to examine the dietary influences in specific cells, tissues, and organisms, and to understand how certain diets influence homeostasis. This knowledge can assist in treating or preventing diseases and parasitic infections. Several mRNA expression research studies have focused on characterization of expression patterns of certain genes involved in absorption and transport of nutrients across the epithelial cell barrier in different segments of the alimentary canal and maintenance of the rumen pH. Liao et al. (2010) found glucose transporter 2 (GLUT2) transports glucose, fructose, and majority of other monosaccharides across the basolateral and apical membranes in the duodenum, and glucose transporter 5 (GLUT5) transports only fructose across the brush border membrane and apical membrane of duodenal enterocytes. Gäbel and Aschenbach (2006) found that the maintenance of rumen intracellular pH is accomplished partly through exchange proteins, such as sodium/hydrogen exchange (NHE) proteins and monocarboxylate transporter isoform 1 (MCT1) that regulate cellular H^+ efflux and transport VFA metabolites into the blood (Muller et al., 2000). Supporting Muller's findings Graham and Simmons (2005a) found MCT1 is located on the basal side of the ruminal epithelial cells and is responsible for the removal of protons from the rumen wall by co-transporter dissociated VFAs as well. Furthermore, Garcia et al. (1994) findings show transportation of short chain fatty acids, lactate, pyruvate, produced during microbial fermentation in the rumen, is performed by MCT 1.

Gaining more information on the specific gene functions throughout the digestion tract would benefit the animal and the producer. The more knowledge nutritionists have on rumen function will allow better utilization of feedstuffs and diet preparation that can improve performance and decrease chances of metabolic disorders.

SUMMARY

To be able to prevent or control acidosis, laminitis, and bloat in the cattle industry would help decrease mortality and health treatment expenses. Concentrating on acidosis would help decrease the occurrence of lameness and bloat. Overall, it would lead to healthier and higher performing cattle. Further research is required to determine how exactly both dairy and beef cattle being introduced to high fiber and concentrate diets supplemented with *Aspergillus oryzae* would respond. Research analyzing the rumen epithelium would give us another factor to critique. As the rumen epithelium plays a major role in the absorption and buffering, its adaptation to high concentrated diets may play a role in alleviating the detrimental effects of acidosis (Steele et al., 2012). Further analysis of specific gene expression in the ruminant gastric intestinal tract in complex studies is needed. Since butyrate is the most extensively metabolized by the rumen epithelium and intracellular pH regulator in the rumen epithelium cells looking for the relationship of acidosis and non-acidosis cattle may give some new insight (Schlau et al., 2012).

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CHAPTER II: Effects of *Aspergillus oryzae* α-amylase supplementation on rumen volatile fatty acid profile and relative abundance of mRNA associated with nutrient transporters in ruminal and duodenal tissue.

SUMMARY

The objective of this study was to investigate the impact of *Aspergillus oryzae* α-amylase (AAM) supplementation on rumen VFA profile and relative abundance of mRNA associated with nutrient absorption in ruminal and duodenal tissue from beef steers. Nine crossbred steers (average BW 622 ± 50 kg), with rumen and duodenal fistulas were housed in individual stations and fed a high concentrate finishing diet twice daily for 8 d. Treatments included CON (5 g corn meal; n= 5) and AAM (5 g of 750 fungal α-amylase units/g; n= 4). Dietary treatment supplements were applied as a top dress (3 g of α-amylase or corn meal into 150 g of dried distiller's grains (DDG) for the AM feeding and 2 g of α-amylase or corn meal into 100 g of DDG for PM feeding). On d 5, rumen fluid samples were obtained every 4 h for 24 h and analyzed for VFA concentration. On d 9, rumen papillae and duodenal mucosal tissue samples were collected. Total tissue RNA was extracted for real-time PCR analysis. Sodium/potassium ATPase pump α1, glucose transporter 2 and 5, putative anion transporter, isoform1, sodium/hydrogen antiporter isoforms1, 2 and 3, 3-hydroxy 3-methylglutaryl coenzyme A synthase isoform2, down regulated in adenoma, monocarboxylate co-transporter isoform1, and glyceraldehyde-3-phosphate dehydrogenase mRNA were tested. Relative expression (fold change) of mRNA in ruminal and duodenal tissues were analyzed using PROC GLM and VFA distribution was analyzed as a randomized block design with repeated measures using the MIXED procedure of SAS. Concentrations of VFA and the acetate to propionate ratio were

similar across treatments. However, acetate:propionate ratio and butyrate molar percentage were numerically greater ($P = 0.17$) in AAM steers compared to controls. Genes tested were not significantly changed by AAM supplementation in the rumen or duodenum. However, genes involved in nutrient absorption were numerically decreased in the rumen and increased in the duodenum in the AAM supplemented steers compared to the controls.

Key words: duodenum, fungal α-amylase, gene, rumen, steer, volatile fatty acids

INTRODUCTION

Starch from cereal grains is the primary dietary energy source for finishing cattle, representing 50 to 70% of the finishing diet. Amylose and amylopectin comprise most of the starch in the finishing beef cattle diet (Huntington, 1997). The rate and extent of starch digestion in the rumen has an impact on total tract starch digestibility and ruminant performance (Lykos et al., 1997). Extensive ruminal starch digestion is beneficial; however rapid starch fermentation could possibly result in acidosis (Owens et al., 1998). Understanding the correct balance for optimizing ruminal starch fermentation while avoiding conditions leading to ruminal upset is desirable for preventing acidosis in beef cattle. With the increasing demand and development of more natural markets, a natural substitute for feed grade antibiotics and ionophores needs to be explored.

Direct- fed microbials (DFM) or probiotics are defined as “a live microbial feed supplement which beneficially affects the host by improving gastrointestinal tract microbial balance” (Heyman and Menard, 2002). Direct-fed microbials for ruminants can be supplied in the form of fungi. *Aspergillus oryzae*, a fungus, produces α-amylase that aids in the digestion of starch. Tricarico et al. (2005) reported that supplemental fungal α-amylase did not increase

ruminal starch digestion but consistently increased butyrate and reduced propionate molar proportions in the rumen, indicating a shift in microbial starch digestion may have happened but not total ruminal starch digestibility. We hypothesized that *A. oryzae* α-amylase supplementation to high concentrate diets fed to finishing steers would increase microbial production of butyric acid and decrease propionate production and therefore increase the relative abundance of mRNA butyric acid transporters in the epithelial wall of the rumen. Therefore the objective of this experiment was to investigate the impact of *A. oryzae* α-amylase supplementation on volatile fatty acid profiles in the rumen and relative abundance of mRNA associated with genes related to nutrient absorption in ruminal and duodenal tissue from beef steers fed a high concentrate steam flaked corn based diet.

MATERIALS AND METHODS

Animal Care

Nine beef steers fitted with rumen and duodenum fistulas were utilized in this study. All animal care and handling described herein were conducted according to the guidelines approved by the Colorado State University Institutional Animal Care and Use Committee. Steers were housed in a climate controlled metabolism building in individual stations equipped with automatic waverers and feed troughs and fed a high concentrate finishing diet (74.6% corn on a DM basis) twice daily for 8 d (Table 1). Treatments included 1) CON (control; 5 g of corn meal) and 2) AAM (α-amylase: Amaize; 5 g of 750 fungal amylase units/g; Alltech Inc. Nicholasville, KY). Each gram of Amaize contained 750 fungal α-amylase units (FAU). Dietary treatment supplements were manufactured prior to each feeding by mixing 3 g of α-amylase or corn meal into 150 g of dried distiller's grains (DDG) for the morning feeding and 2 g of α-amylase (per animal) or corn meal into 100 g DDG for the afternoon feeding. Supplements were applied as a

top dress to each feed bunk for every feeding and thoroughly mixed by hand. On d 5, rumen fluid samples were obtained every 4 h for 24 h and analyzed for VFA. On d 9, rumen and duodenal epithelial biopsy samples were collected as described below. Total tissue RNA was extracted from both tissues and relative expression of mRNA was determined for sodium/potassium ATPase pump, α 1 (ATP1), glucose transporters 2 and 5 (GLUT2, GLUT5), putative anion transporter, isoform 1 (PAT1), sodium/hydrogen antiporter, isoform 1, 2 and 3 (NHE1, NHE2, and NHE3), 3-hydroxy 3-methylglutaryl coenzyme A synthase isoform 2 (HMGCS2), down regulated in adenoma (DRA), monocarboxylate co-transporter, isoform 1 (MCT1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for assay normalization. Primer sequences for quantitative real-time PCR analysis of selected genes appear in Table 2.

Ruminal Papillae and Duodenal Mucosal Collection

On d 6 and 7, Meloxicam (1.0 mg/kg BW, 0.5mg/kg BW; respectively) was administered to each steer. On d 9, steers were fed two hours prior to ruminal and duodenal tissue biopsies. For the biopsy procedure, steers were transported to the veterinary teaching hospital at Colorado State University. Once at the hospital each steer was weighed and given a single dose of Oxytetracycline (200mg/mL, 20mg/kg SQ). An 8 mm diameter skin biopsy instrument was used to collect approximately 10 rumen papillae pinch biopsies from an area (approximately 100 cm^2) from the rumen caudal wall. Once obtained, the biopsy tissue was rinsed immediately with sterilized phosphate buffered solution (pH 7.4), treated with RNA later solution(Qiagen, Valencia, CA, USA) and snap frozen in liquid N_2. Samples were stored at -80°C. Duodenal mucosal biopsies were obtained immediately post ruminal papillae biopsies. Briefly duodenal mucosal samples were obtained using a sterile medical spatula scraper approximately 100 cm

aboral to the abomasal sphincter. Initially, duodenal contents were aspirated and the medical spatula was inserted into the duodenal fistula parallel to the intestinal lining approximately 15 cm into the duodenum aborally. Gentle pressure was applied with spatula tip to the mucosal lining of the intestine as the spatula was withdrawn. Tissue from the spatula was collected and treated as described for the rumen papillae pinch biopsies.

Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis

Total tissue RNA was extracted from 100 mg of both ruminal and duodenal tissue samples by homogenizing in 1 ml Tri Reagent (Sigma, St. Louis, MO, USA). Homogenates were then incubated at room temperature for 10 min, mixed with chloroform (0.2 mL), and incubated at room temperature for an additional 15 min followed by centrifugal separation at 13,362.34 g force for 15 min at 4ºC. The RNA in the supernatant was precipitated by 0.5 mL of isopropanol and incubated for 10 min at room temperature. Ribonucleic acid was pelleted by centrifugation at 13,362.34 g force for 15 min at 4ºC. Pellets were re-suspended and purified using RNEasy Mini kit (Qiagen, Valencia, CA, USA) with RNase-free DNaseI (Qiagen, Valencia, CA, USA) treatment to remove possible DNA contamination. The RNA concentration of the extract obtained was determined using a NanoDropND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at absorbances of 260 and 280 nm. Single stranded complementary DNA were synthesized from total cellular RNA using iScript Reverse Transcriptase (BioRad, Hercules, CA, USA) at 25ºC for 5 min, 42ºC for 30 min, and 85ºC for 5 min incubation. Products were diluted 10 X with DNAse/RNAse free water.

Semi-quantitative real-time PCR

Complementary DNA was used as a template for semi-quantitative Real Time PCR. Target genes and GAPDH were amplified using SYBR Green (BioRad, Hercules, CA, USA).

Real-time PCR for target genes and GAPDH amplification was conducted using the iQ5 real-time PCR system (Bio-Rad, Hercules, CA, USA) with 96-well plates. Polymerase chain reaction cycle conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for15 s followed by a melt curve analysis to confirm amplification of single cDNA products. After amplification, products were melted to ensure the quality of amplification. The RT-PCR products were resolved as a single band using agarose gel electrophoresis. Bands from agarose gels were excised, purified and sequenced to confirm identity with each target gene. Each target gene expression was determined relative to GAPDH using delta-delta method (Schmittgen and Livak, 2008).

Ruminal Volatile Fatty Acids Collection

Quadruplicate 200 mL samples of fluid were removed from the geometric center of the rumen. Two sub-samples were stored for VFA analysis. Volatile fatty acid concentrations were determined via FID gas chromatography as described by Erwin et al. (1961).

Statistical analysis

Statistical analysis for the randomized block design with two treatments was carried out using SAS (version 9.3; SAS Institute Inc., Cary, NC). Relative expression (fold change) of mRNA in ruminal and duodenal tissues were analyzed using PROC GLM of SAS. Classification variables included animal and treatment. Treatment was included in the model as a fixed class variable. Differences between treatments means were determine by utilizing LSMEANS TRT/PDIFF STDERR statement. Ruminal VFA concentrations were analyzed as a randomized block design with repeated measures using the MIXED procedure of SAS and repeated measures where appropriate using time as the experimental unit. Classification variables included steer, treatment, and time. Treatment was included in the model as a fixed class variable, where steer

was included in the model as a random class variable and time was considered the experimental unit. Differences between treatments means were determine by utilizing LSMEANS TRT TIME TREATMENT*TIME/PDIFF statement.

RESULTS AND DISCUSSION

Ruminal Volatile Fatty Acids

Rumen VFA concentrations are reported in Table 3. There were no interactions between treatment and time for any of the VFAs measured, therefore data are expressed as overall means. As expected VFA concentrations and the acetate:propionate ratio were similar to VFA profiles of cattle fed a steam-flaked corn based finishing diet (May et al., 2009). Volatile fatty acid concentrations and the acetate to propionate ratio were similar across treatments. However, butyrate concentration was numerically ($P < 0.18$) greater in AAM steers compared to CON steers (12.06% vs. 8.96%, respectively). Also acetate:propionate molar proportions numerically increased (11%) in AAM steers compared to CON steers. These numerical differences are comparable to data reported by Tricarico et al. (2005) in lactating dairy cows. Tricarico et al. (2005) found that AAM supplementation increased the molar proportions of acetate and butyrate, and reduced the molar proportion of propionate compared to control animals, and consequently, the acetate to propionate ratio was greater in cows supplemented with AAM when compared to controls.

Gene expression

Rumen Papillae: Relative expression of mRNA associated with specific genes in the rumen papillae and duodenum are presented in Table 4 and 5. Similar to VFA profile in the rumen fluid, dietary supplementation of AAM did not influence relative expression of mRNA quantified in ruminal or duodenal epithelium. While not statistically different, mRNA's that

were involved in nutrient absorption (GLUT 2, PAT1, DRA and MCT1) and in acid neutralization as well as VFA absorption (NHEs) were down regulated in the rumen epithelium while upregulation occurred in duodenal epithelium in AAM supplemented steers compared to controls.

Glucose transporter 2 transports glucose, fructose, and majority of other monosaccharides across the basolateral and apical membranes in the duodenum, and GLUT5 transports only fructose across the brush border membrane and apical membrane of duodenal enterocytes (Liao et al., 2010). Monocarboxylate co-transporterisoform1 is located on the basal side of the ruminal epithelial cells and is responsible for the removal of protons from the rumen wall by co-transporter dissociated VFAs (Graham and Simmons, 2005b). Furthermore, MCT1 is responsible for transport of monocarboxylates such as lactate and pyruvate across the rumen wall into the circulatory system (Garcia et al., 1994). Down regulated in adenoma and PAT1 in the rumen may help in VFA absorption through the rumen wall and export of bicarbonate in exchange (Bilk et al., 2005).

While none of the genes above were differently expressed, lower expression of these nutrient transporters tested suggests that supplementation of AAM in the diet may not improve starch digestion in the rumen. This is also supported by down regulation of sodium hydrogen antiporters (NHEs). On the other hand, numerical increase of GLUT2 (46%) and MCT1 (102%) mRNAs in the duodenum of AAM supplemented steers suggests there was an increase in nutrient absorption in the small intestine. Glucose transporter 2 facilitates absorption of simple sugars in the luminal epithelium in the small intestine. Transportation of short chain fatty acids, lactate, pyruvate, produced during microbial fermentation in the rumen, is performed by MCT 1(Garcia et al., 1994). However, almost all VFAs are absorbed across the rumen wall and

minimal amounts enter the duodenum (Peters et al., 1990). Therefore, MCT1 may contribute neutralization of the small intestine rather than VFA absorption in the small intestine.

Supplementation of yeast culture (*Saccharomyces cerevisiae*) improved apparent ruminal digestion while fungal culture (*A. oryzae*) did not change the ruminal digestion in dairy cows (Yoon and Stern, 1996). However, apparent total tract digestion did not differ between yeast culture and fungal culture supplementation. This result suggests that AAM may improve the intestinal digestion rather than ruminal fermentation. The data from the current experiment agrees with others that AAM may simulate nutrient digestion/absorption towards small intestine rather than in the rumen. While it was not significant, mRNA expression profile supports the shift of the nutrient fermentation/absorption. The group of genes examined is involved in the nutrient transport mechanism. While it was not significantly changed by supplementation of α-amylase, gene expression levels were numerically lower in the rumen and higher in the duodenum when compared to control groups. While the influence of addition of α-amylase in the flaked corn based finishing diet did not influence the fermentation production transporter gene expression profile, it is noteworthy that addition of α-amylase in the diet increased the expression of a few genes numerically.

However, it is worth to note that enhance of small intestine starch digestion may not be a direct effect of *A. oryzae* α-amylase. Low pH at abomasum (~pH 2.0) probably inactivates the AAM enzyme activity before it reaches to the small intestine. Rather, improvement of small intestine starch digestion could be stimulation of pancreatic amylase secretion. As reported by others, addition of AAM increases the butyrate production in the rumen. Most of the butyrate absorbed by ruminal epithelial cell is utilized by the cell itself for growth (Britton and Krehbiel, 1993; Kristensen and Harmon, 2004). Growth of ruminal papillae can result in increased

ruminal VFA absorption which could stimulate pancreatic amylase secretion into small intestine. Further research including a wider selection of genes is needed to help understand the impact of Amaize on nutrient digestion in finishing feedlot steers.

Due to its difficulty of tissue collection in the rumen and small intestine, there are limited published data available (Komatsu et al., 2005; Schlau et al., 2012). Under the conditions of this experiment these data indicate that genes involved in nutrient absorption in the rumen papillae and duodenal mucosa were not influenced by dietary treatment. Although butyrate tended to be higher in rumen fluid of steers supplemented with AAM genes responsible for nutrient absorption were not influenced. Addition of AAM in steam flaked corn based finishing diets does not appear to influence the ruminal digestibility of nonstructural carbohydrates. However, AAM supplementation may influence butyrate production in the rumen.

Table 1. Dry matter composition of diets for the steers supplemented with *Aspergillus oryzae* α-amylase.

Item[1]	Starter	Step-up 1	Step-up 2	Finish
Ingredient				
Corn silage	23.74	31.80	17.95	14.63
Alfalfa hay	27.97	13.65	9.10	
Steam flaked corn	33.78	40.21	61.45	74.55
DDG[2]	11.74	10.22	6.20	4.02
CSU Beef R650[3]	2.14	2.86	3.57	4.29
Limestone	0.62	1.03	1.30	1.62
Urea		0.23	0.37	0.52
Potassium chloride				0.37
Theoretical Nutrients				
Dry matter	65.07	59.46	68.990	71.40
Crude protein	13.50	13.50	13.50	
Non-protein nitrogen[4]	1.02	2.00	2.75	3.50
NEm, Mcal/kg	0.78	0.82	0.89	0.94
NEg, Mcal/kg	0.50	0.53	0.59	0.64
Acid detergent fiber	19.68	16.65	11.21	7.07
Neutral detergent fiber	32.63	28.58	20.70	14.98
fNDF[5]	24.00	20.00	12.00	6.00
Crude fat	4.07	4.23	4.19	4.27
Calcium	0.70	0.70	0.70	0.70
Phosphorus	0.34	0.34	0.32	0.31
Potassium	0.99	0.88	0.70	0.70
Magnesium	0.22	0.21	0.19	0.17
Salt	0.38	0.51	0.66	0.94
Sulfur	0.20	0.18	0.16	0.15
Vitamin A, IU/lb DM	1447.09	1929.45	2411.80	2894.17
Vitamin E, IU/lb DM	2.10	2.80	3.50	4.20
Monensin, g/ton DM	15.00	20.00	25.00	30.00

[1] Percentage of dry matter.
[2] Dried distiller's grains plus solubles.
[3] Manufactured by Agfinity, Inc., P. O. Box 338, Eaton, CO.
[4] Crude protein equivalent.
[5] NDF from the forage component of the diet.

Table 2. Primer and probe sequences and National Center for Biotechnology Information accession numbers for quantitative real-time PCR analysis.

Gene Name[1]	Accession Number	Primer sequences
HMGCS2	NM_001045883.1	Forward: ACTGCCCTCCCTCTTCAATTGC Reverse: ATTTGTCCAGGGCCCGTAAG
ATP1A1	NM_001076798.1	Forward: GGAAGGGGGGTTGGACGTGAT Reverse: TTCCACCAGATCTCCGACGA
NHE1	NM_174833.2	Forward: GCCTTAGCAAGAGTCCAGCA Reverse: AGGAATGGTTAACAGGGCGG
NHE2	XM_604493.6	Forward: GTTGACGTGTTTGCTGGCAT Reverse: GGAACACTGAGGACGGAAGG
NHE3	AJ131764.1	Forward: GACCCCAAGCTCAACGAGAA Reverse: TCTCGAGACTTCTGAGCCGA
NHE1s	NM_174833.2	Forward: ACGCCTTAGCAAGAGTCCAG Reverse: CAAACTGGGGAAGAGGGACC
PAT1	BC_123616.1	Forward: TTTGCCATCGCCATCTCACT Reverse: AGGGAAGCACTGGAAGATGC
DRA	NM_001083676.1	Forward: CAGTTGCTGATGGGGGTTCT Reverse: ATTGGGATGGGCACTGGAAG
MCT1	NM_001037319.1	Forward: GTGCTCGGACGAGCGAACTA Reverse: ATCATGACTGGACGACTGCC
GAPDH	NM_001034034.2	Forward: GGTTGTCTCCTGCGACTTCA Reverse: TTCTCAGTGTGGCGGAGATG
GLUT2	XM_614140.3	Forward: GGCTTAGCAGGGAGCTGAAAA Reverse: CTGGAACAGCTAAGGGGACA
GLUT5	NM_00110142.1	Forward: TCCAGAGCAAAGATGGAGCC Reverse: GAAGCCTCCGAAGGGAAACA

[1]HMGCS2: 3-Hydroxy 3-methylglutaryl coenzyme A synthase isoform 2; ATP1: Sodium/potassium ATPase pump, α 1; NHE1, 2, 3: Sodium/hydrogen antiporter, isoform 1, 2, 3; NHE1s: Sodium/hydrogen antiporter, isoform 1s; PAT1: Putative anion transporter, isoform 1; DRA: Down regulated in adenoma; MCT1: Monocarboxylate co-transporter, isoform1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GLUT2: Glucose transporter 2; GLUT5: Glucose transporter 5;

Table 3. Molar percentage of **r**uminal Volatile Fatty Acid concentrations of the steers supplemented with A. oryzae α-amylase.

	Dietary Treatment			Contrasts (P<)		
Volatile Fatty Acid	CON[1]	AAM[2]	SEM	Trt	Time	Trt * Time
Butyrate	8.96	12.06	1.56	0.1702	0.9008	0.8613
Propionate	36.96	33.68	1.87	0.2251	0.0001	0.7373
Acetate: Propionate	1.35	1.5	0.1	0.3162	0.0001	0.6918
Valerate	2.98	2.58	0.42	0.5045	0.2255	0.7693
Isovalerate	1.56	1.88	0.55	0.686	0.0001	0.0862
Isobutyrate	0.67	0.72	0.16	0.7981	0.0001	0.5861
Acetate	48.88	49.08	1.32	0.9161	0.0001	0.3021

[1]Control group
[2]Amaize group

Table 4. Relative expression (fold change) of mRNA in the rumen of steers supplemented with A. oryzae α-amylase.

	Rumen				
Gene[1]	CON[2]	AAM[3]	SEM	% change	*P* <
NHE1	4.2	2.6	0.71	-38.7	0.17
PAT1[4]	2.3	1.4	0.40	-37.6	0.17
DRA[5]	4.4	2.1	1.06	-51.7	0.18
NHE2	12.4	6.4	3.23	-48.2	0.24
GLUT2	951.6	315.0	446.30	-66.9	0.35
MCT1	3.3	4.1	0.81	27.4	0.46
GLUT5	254.2	186.4	71.70	-26.7	0.53
NHE3	5.2	4.4	0.98	-15.3	0.58
ATP1A1	1.3	1.6	0.55	20.8	0.74
HMGCS2	3.8	3.2	1.32	-16.2	0.75
NHE1s	2.9	2.6	1.00	-11.3	0.82

[1] NHE1, 2, 3: Sodium/hydrogen antiporter, isoform 1, 2, 3; PAT1: Putative anion transporter, isoform 1; DRA: Down regulated in adenoma; GLUT2: Glucose transporter 2; MCT1: Monocarboxylate co-transporter, isoform1; GLUT5: Glucose transporter 5; ATP1A1: Sodium/potassium ATPase pump, α 1; HMGCS2: 3-Hydroxy 3-methylglutaryl coenzyme A synthase isoform 2; NHE1s: Sodium/hydrogen antiporter, isoform 1s.
[2]Control group
[3]Amaize group
[4]SLC26A6
[5]SLC26A3

Table 5. Relative expression (fold change) of mRNA in the duodenum of steers supplemented with A. oryzae α-amylase.

	Duodenum				
Gene[1]	CON[2]	AAM[3]	SEM	% change	*P* <
NHE1	2.7	5.1	1.61	87.2	0.34
PAT1[4]	3.2	2.5	0.75	-20.8	0.55
DRA[5]	7.0	23.6	11.23	238.6	0.33
NHE2	8.0	8.9	4.0	10.9	0.88
GLUT2	2.2	3.2	0.95	46.3	0.48
MCT1	6.1	12.3	4.0	101.6	0.31
GLUT5	3.0	3.1	0.78	4.0	0.92
NHE3	2.8	2.6	0.73	-5.0	0.89
ATP1A1	2.7	4.4	1.46	63.3	0.43
HMGCS2	14.7	16.8	4.37	14.5	0.74
NHE1s	2.4	2.9	0.94	22.8	0.69

[1] NHE1, 2, 3: Sodium/hydrogen antiporter, isoform 1, 2, 3; PAT1: Putative anion transporter, isoform 1; DRA: Down regulated in adenoma; GLUT2: Glucose transporter 2; MCT1: Monocarboxylate co-transporter, isoform1; GLUT5: Glucose transporter 5; ATP1A1: Sodium/potassium ATPase pump, α 1; HMGCS2: 3-Hydroxy 3-methylglutaryl coenzyme A synthase isoform 2; NHE1s: Sodium/hydrogen antiporter, isoform 1s.
[2]Control group
[3]Amaize group
[4]SLC26A6
[5]SLC26A3

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APPENDIX I

BATCH SHEETS

Table 6. Batch sheets for the steers supplemented with *Aspergillus oryzae* α-amylase.

Ingredient[1]	Starter	Step 1	Step 2	Finish
Corn silage	221	270	177	149
Alfalfa hay	100	45	34	
Steam flaked corn	128	139	247	310
DDG[2]	42	33	23	16
CSU Beef R650[3]	8	9	13	17
Limestone	2	3	5	6
Urea		1	1	2
Potassium chloride				1

[1] LB, as-fed basis.
[2] Dried distiller's grains plus solubles.
[3] Manufactured by Agfinity, Inc., P. O. Box 338, Eaton, CO.

APPENDIX II

Relative expression (fold change) of mRNA in rumen tissue samples data.

Relative expression (fold change) of mRNA in rumen tissue samples data.

Obs	Tissue	Animal	Trt	GLUT5	PAT1	NHE3	NHE1	RPLPO	HMGCS2	GLUT2	DRA	ACTbeta	MCT1	NHE2	ATP1	NHE1S
1	Rumen	4	A	456.67	1.14	6.34	3.85	1.28	2.06	913.33	1.74	6.13	3.71	7.75	0.69	1.00
2	Rumen	6	A	135.77	1.00	4.11	1.79	1.02	2.86	180.39	2.70	4.59	5.46	3.81	1.14	1.99
3	Rumen	10	A	1.00	1.80	1.00	1.00	1.06	1.00	1.00	1.00	4.52	4.69	11.88	0.61	.
4	Rumen	1	A	152.22	1.78	6.30	3.63	1.85	6.66	165.42	2.96	3.11	2.71	2.15	3.85	4.74
5	Rumen	2	C	215.27	2.05	4.29	5.41	1.18	2.91	216.77	2.28	5.43	5.52	19.36	1.13	1.69
6	Rumen	3	C	334.30	3.71	6.52	2.96	1.59	2.37	867.07	5.43	4.52	2.47	12.77	1.69	3.19
7	Rumen	8	C	275.33	1.24	4.29	4.21	1.97	7.92	2665.15	7.89	6.61	4.00	16.28	1.00	1.44
8	Rumen	5	C	192.00	2.15	5.86	.	1.00	1.83	57.48	1.80	1.00	1.00	1.00	1.39	5.33

Relative expression (fold change) of mRNA in duodenum mucosal tissue samples data.

Relative expression (fold change) of mRNA in duodenum mucosal tissue samples data.

Obs	Tissue	Animal	Trt	GLUT5	PAT1	NHE3	NHE1	RPLPO	HMGCS2	GLUT2	DRA	ACTbeta	MCT1	NHE2	ATP1	NHE1S
1	Duodenum	4	A	3.26	1.46	4.07	2.57	2.21	27.76	7.46	76.37	13.36	28.15	15.94	3.28	2.01
2	Duodenum	6	A	2.81	3.14	1.42	6.52	1.61	1.00	1.75	8.28	6.70	13.09	1.54	1.66	1.00
3	Duodenum	10	A	1.00	1.00	1.60	1.00	1.00	13.50	1.97	2.29	8.37	4.69	16.68	2.47	2.04
4	Duodenum	1	A	5.41	4.48	3.46	10.34	7.06	25.11	1.59	7.46	2.04	3.17	1.23	10.23	6.61
5	Duodenum	2	C	4.41	2.69	3.14	5.24	2.00	20.61	1.57	11.71	5.45	13.13	20.39	1.24	1.91
6	Duodenum	3	C	1.62	2.39	1.00	1.32	2.42	5.64	3.10	17.03	11.12	4.55	4.89	1.00	.
7	Duodenum	8	C	1.32	1.68	1.65	1.77	1.55	12.95	3.22	1.00	9.55	9.45	12.42	1.24	1.41
8	Duodenum	5	C	4.47	5.82	5.35	.	2.51	19.90	1.00	3.19	1.00	1.00	1.00	3.97	2.91
9	Duodenum	9	C	3.18	3.34	2.78	2.58	3.32	14.47	2.01	1.90	2.30	2.31	1.19	6.04	3.24

Volatile fatty acid distribution data.

Volatile fatty acid distribution data

Obs	steer	time	trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Ratio	Total	time1
1	1	0	2	43.16	37.09	1.07	12.87	3.16	2.64	1.16	100	0
2	2	0	1	53.04	34.03	0.70	7.01	1.21	4.02	1.56	100	0
3	3	0	1	51.56	38.99	0.51	5.77	1.17	1.99	1.32	100	0
4	4	0	2	49.14	27.33	0.76	18.04	2.73	1.99	1.80	100	0
5	5	0	1	48.91	31.66	0.83	11.62	2.60	4.38	1.54	100	0
6	6	0	2	49.76	40.18	0.37	6.91	0.50	2.27	1.24	100	0
7	8	0	1	45.62	40.34	0.76	8.76	2.03	2.49	1.13	100	0
8	9	0	1	53.73	31.14	1.20	9.35	2.21	2.37	1.73	100	0
9	10	0	2	54.62	28.85	0.75	11.55	0.85	3.39	1.89	100	0
10	1	2	2	43.18	36.79	1.08	13.02	3.23	2.70	1.17	100	2
11	2	2	1	51.81	35.87	0.52	7.03	0.82	3.94	1.44	100	2
12	3	2	1	50.99	39.83	0.38	5.89	0.91	1.99	1.28	100	2
13	4	2	2	48.37	29.84	0.57	17.14	2.31	1.76	1.62	100	2
14	5	2	1	46.24	33.41	0.75	12.87	2.30	4.43	1.38	100	2
15	6	2	2	50.17	38.75	0.20	7.92	0.32	2.64	1.29	100	2
16	8	2	1	43.55	44.35	0.54	8.05	1.42	2.09	0.98	100	2
17	9	2	1	52.62	33.96	0.69	9.22	1.13	2.38	1.55	100	2
18	10	2	2	53.44	32.07	0.42	10.47	0.41	3.18	1.67	100	2
19	1	4	2	43.91	35.11	1.29	12.96	3.88	2.85	1.25	100	4
20	2	4	1	50.81	36.16	0.53	7.55	0.90	4.05	1.41	100	4
21	3	4	1	49.36	40.92	0.40	6.30	1.05	1.97	1.21	100	4
22	4	4	2	45.61	34.18	0.55	15.75	2.13	1.78	1.33	100	4
23	5	4	1	44.14	34.71	0.59	14.10	1.83	4.63	1.27	100	4
24	6	4	2	47.91	39.17	0.17	9.25	0.23	3.28	1.22	100	4
25	8	4	1	42.71	45.83	0.53	7.42	1.36	2.15	0.93	100	4
26	9	4	1	49.66	38.45	0.50	8.66	0.74	1.99	1.29	100	4
27	10	4	2	48.72	37.29	0.35	9.95	0.39	3.29	1.31	100	4
28	1	9	2	48.99	31.49	1.53	10.96	4.39	2.63	1.56	100	9
29	2	9	1	51.25	35.26	0.65	7.66	1.26	3.92	1.45	100	9
30	3	9	1	48.64	41.58	0.53	6.11	1.34	1.79	1.17	100	9
31	4	9	2	46.12	31.55	0.57	17.26	2.12	2.38	1.46	100	9
32	5	9	1	43.92	33.55	0.54	14.30	1.81	5.87	1.31	100	9
33	6	9	2	47.67	40.38	0.31	8.55	0.46	2.64	1.18	100	9
34	8	9	1	44.40	44.52	0.47	7.32	1.21	2.08	1.00	100	9
35	9	9	1	48.95	39.17	0.45	8.46	0.73	2.23	1.25	100	9
36	10	9	2	48.93	35.84	0.36	10.78	0.44	3.64	1.37	100	9
37	1	12	2	51.19	30.30	1.46	10.13	4.53	2.39	1.69	100	12
38	2	12	1	52.24	35.24	0.45	7.79	0.86	3.42	1.48	100	12
39	3	12	1	49.02	41.12	0.32	6.78	0.93	1.83	1.19	100	12
40	4	12	2	46.07	33.63	0.43	16.12	1.86	1.88	1.37	100	12
41	5	12	1	41.91	34.50	0.48	15.57	1.55	6.00	1.22	100	12
42	6	12	2	47.34	39.72	0.14	9.32	0.21	3.26	1.19	100	12
43	8	12	1	42.67	46.68	0.43	7.21	1.11	1.90	0.91	100	12
44	9	12	1	46.27	42.16	0.43	8.57	0.70	1.86	1.10	100	12
45	10	12	2	49.14	36.25	0.32	10.58	0.44	3.27	1.36	100	12
46	1	24	2	56.41	24.95	1.94	9.39	5.13	2.18	2.26	100	24
47	2	24	1	54.36	29.52	1.08	9.30	2.69	3.05	1.84	100	24
48	3	24	1	52.11	32.90	1.19	8.38	3.20	2.22	1.58	100	24
49	4	24	2	49.91	22.98	1.25	20.26	3.37	2.23	2.17	100	24
50	5	24	1	51.25	30.53	0.99	10.43	2.45	4.35	1.68	100	24
51	6	24	2	52.63	36.27	0.45	8.01	0.73	1.91	1.45	100	24
52	8	24	1	50.44	32.53	1.28	10.57	3.04	2.13	1.55	100	24
53	9	24	1	54.20	29.79	1.26	10.68	2.19	1.86	1.82	100	24
54	10	24	2	55.49	28.32	1.01	12.24	1.21	1.73	1.96	100	24

APPENDIX III

Relative expression (fold change) of mRNA in rumen tissue samples data.

Rumen	4	A	456.67	1.14	6.34	3.85	1.28	2.06	913.33	1.74	6.13	3.71
	7.75	0.69	1.00									
Rumen	6	A	135.77	1.00	4.11	1.79	1.02	2.86	180.39	2.70	4.59	5.46
	3.81	1.14	1.99									
Rumen	10	A	1.00	1.80	1.00	1.00	1.06	1.00	1.00	1.00	4.52	4.69
	11.88	0.61	.									
Rumen	1	A	152.22	1.78	6.30	3.63	1.85	6.66	165.42	2.96	3.11	2.71
	2.15	3.85	4.74									
Rumen	2	C	215.27	2.05	4.29	5.41	1.18	2.91	216.77	2.28	5.43	5.52
	19.36	1.13	1.69									
Rumen	3	C	334.30	3.71	6.52	2.96	1.59	2.37	867.07	5.43	4.52	2.47
	12.77	1.69	3.19									
Rumen	8	C	275.33	1.24	4.29	4.21	1.97	7.92	2665.15		7.89	6.61
	4.00	16.28	1.00	1.44								
Rumen	5	C	192.00	2.15	5.86	.	1.00	1.83	57.48	1.80	1.00	1.00
	1.00	1.39	5.33									

Relative expression (fold change) of mRNA in duodenum mucosal tissue samples.

Duodenum	4	A	3.26	1.46	4.07	2.57	2.21	27.76	7.46	76.37	13.36
28.15	15.94	3.28	2.01								
Duodenum	6	A	2.81	3.14	1.42	6.52	1.61	1.00	1.75	8.28	6.70
13.09	1.54	1.66	1.00								
Duodenum	10	A	1.00	1.00	1.60	1.00	1.00	13.50	1.97	2.29	8.37
4.69	16.68	2.47	2.04								
Duodenum	1	A	5.41	4.48	3.46	10.34	7.06	25.11	1.59	7.46	2.04
3.17	1.23	10.23	6.61								
Duodenum	2	C	4.41	2.69	3.14	5.24	2.00	20.61	1.57	11.71	5.45
13.13	20.39	1.24	1.91								
Duodenum	3	C	1.62	2.39	1.00	1.32	2.42	5.64	3.10	17.03	11.12
4.55	4.89	1.00	.								
Duodenum	8	C	1.32	1.68	1.65	1.77	1.55	12.95	3.22	1.00	9.55
9.45	12.42	1.24	1.41								
Duodenum	5	C	4.47	5.82	5.35	.	2.51	19.90	1.00	3.19	1.00
1.00	1.00	3.97	2.91								
Duodenum	9	C	3.18	3.34	2.78	2.58	3.32	14.47	2.01	1.90	2.30
2.31	1.19	6.04	3.24								

Volatile fatty acid distribution data.

1	0	2	43.16	37.09	1.07	12.87	3.16	2.64	1.16	100.0
2	0	1	53.04	34.03	0.70	7.01	1.21	4.02	1.56	100.0
3	0	1	51.56	38.99	0.51	5.77	1.17	1.99	1.32	100.0
4	0	2	49.14	27.33	0.76	18.04	2.73	1.99	1.80	100.0
5	0	1	48.91	31.66	0.83	11.62	2.60	4.38	1.54	100.0
6	0	2	49.76	40.18	0.37	6.91	0.50	2.27	1.24	100.0
8	0	1	45.62	40.34	0.76	8.76	2.03	2.49	1.13	100.0
9	0	1	53.73	31.14	1.20	9.35	2.21	2.37	1.73	100.0
10	0	2	54.62	28.85	0.75	11.55	0.85	3.39	1.89	100.0
1	2	2	43.18	36.79	1.08	13.02	3.23	2.70	1.17	100.0
2	2	1	51.81	35.87	0.52	7.03	0.82	3.94	1.44	100.0
3	2	1	50.99	39.83	0.38	5.89	0.91	1.99	1.28	100.0
4	2	2	48.37	29.84	0.57	17.14	2.31	1.76	1.62	100.0
5	2	1	46.24	33.41	0.75	12.87	2.30	4.43	1.38	100.0
6	2	2	50.17	38.75	0.20	7.92	0.32	2.64	1.29	100.0
8	2	1	43.55	44.35	0.54	8.05	1.42	2.09	0.98	100.0
9	2	1	52.62	33.96	0.69	9.22	1.13	2.38	1.55	100.0
10	2	2	53.44	32.07	0.42	10.47	0.41	3.18	1.67	100.0
1	4	2	43.91	35.11	1.29	12.96	3.88	2.85	1.25	100.0
2	4	1	50.81	36.16	0.53	7.55	0.90	4.05	1.41	100.0
3	4	1	49.36	40.92	0.40	6.30	1.05	1.97	1.21	100.0
4	4	2	45.61	34.18	0.55	15.75	2.13	1.78	1.33	100.0
5	4	1	44.14	34.71	0.59	14.10	1.83	4.63	1.27	100.0
6	4	2	47.91	39.17	0.17	9.25	0.23	3.28	1.22	100.0
8	4	1	42.71	45.83	0.53	7.42	1.36	2.15	0.93	100.0
9	4	1	49.66	38.45	0.50	8.66	0.74	1.99	1.29	100.0
10	4	2	48.72	37.29	0.35	9.95	0.39	3.29	1.31	100.0
1	9	2	48.99	31.49	1.53	10.96	4.39	2.63	1.56	100.0
2	9	1	51.25	35.26	0.65	7.66	1.26	3.92	1.45	100.0
3	9	1	48.64	41.58	0.53	6.11	1.34	1.79	1.17	100.0
4	9	2	46.12	31.55	0.57	17.26	2.12	2.38	1.46	100.0
5	9	1	43.92	33.55	0.54	14.30	1.81	5.87	1.31	100.0
6	9	2	47.67	40.38	0.31	8.55	0.46	2.64	1.18	100.0
8	9	1	44.40	44.52	0.47	7.32	1.21	2.08	1.00	100.0
9	9	1	48.95	39.17	0.45	8.46	0.73	2.23	1.25	100.0
10	9	2	48.93	35.84	0.36	10.78	0.44	3.64	1.37	100.0
1	12	2	51.19	30.30	1.46	10.13	4.53	2.39	1.69	100.0
2	12	1	52.24	35.24	0.45	7.79	0.86	3.42	1.48	100.0
3	12	1	49.02	41.12	0.32	6.78	0.93	1.83	1.19	100.0
4	12	2	46.07	33.63	0.43	16.12	1.86	1.88	1.37	100.0
5	12	1	41.91	34.50	0.48	15.57	1.55	6.00	1.22	100.0
6	12	2	47.34	39.72	0.14	9.32	0.21	3.26	1.19	100.0
8	12	1	42.67	46.68	0.43	7.21	1.11	1.90	0.91	100.0
9	12	1	46.27	42.16	0.43	8.57	0.70	1.86	1.10	100.0
10	12	2	49.14	36.25	0.32	10.58	0.44	3.27	1.36	100.0

1	24	2	56.41	24.95	1.94	9.39	5.13	2.18	2.26	100.0
2	24	1	54.36	29.52	1.08	9.30	2.69	3.05	1.84	100.0
3	24	1	52.11	32.90	1.19	8.38	3.20	2.22	1.58	100.0
4	24	2	49.91	22.98	1.25	20.26	3.37	2.23	2.17	100.0
5	24	1	51.25	30.53	0.99	10.43	2.45	4.35	1.68	100.0
6	24	2	52.63	36.27	0.45	8.01	0.73	1.91	1.45	100.0
8	24	1	50.44	32.53	1.28	10.57	3.04	2.13	1.55	100.0
9	24	1	54.20	29.79	1.26	10.68	2.19	1.86	1.82	100.0
10	24	2	55.49	28.32	1.01	12.24	1.21	1.73	1.96	100.0

APPENIX IV

SAS CODE

Analyze relative expression (fold change) of mRNA in rumen tissue samples data.

```
DM log "OUT;CLEAR;LOG;CLEAR;";
DM log'next results; clear; cancel' whostedit continue;

options ls=100 ps=150;

data Fistulated Steers;
input Tissue $ Animal Trt $ GLUT5  PAT1   NHE3  NHE1  RPLPO          HMGCS2
          GLUT2          DRA    ACTbeta        MCT1 NHE2  ATP1   NHE1S;
cards;


Title 'Steer Rumen Genes Fold';
run;


proc GLM;
class animal trt;
model GLUT5 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model PAT1 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE3 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE1 = trt;
lsmeans  trt/pdiff stderr;
```

```
run;

proc GLM;
class animal trt;
model RPLPO = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model HMGCS2 = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model GLUT2 = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model DRA = trt;
lsmeans  trt/pdiff stderr;
run;
proc GLM;
class animal trt;
model ACTbeta = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model MCT1 = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model NHE2 = trt;
```

```
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model ATP1 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE1s = trt;
lsmeans  trt/pdiff stderr;
run;
quit;
```

Analyze relative expression (fold change) of mRNA in duodenum mucosal tissue samples data.

```
DM log "OUT;CLEAR;LOG;CLEAR;";
DM log'next results; clear; cancel' whostedit continue;

options ls=100 ps=150;

data Fistulated Steers;
input Tissue $ Animal Trt $ GLUT5  PAT1   NHE3  NHE1  RPLPO          HMGCS2
          GLUT2          DRA    ACTbeta        MCT1 NHE2  ATP1   NHE1S;
cards;

Title 'Steer Duodenum Genes Fold';
run;


proc GLM;
class animal trt;
model GLUT5 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model PAT1 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE3 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE1 = trt;
lsmeans  trt/pdiff stderr;
run;
```

```
proc GLM;
class animal trt;
model RPLPO = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model HMGCS2 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model GLUT2 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model DRA = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model ACTbeta = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model MCT1 = trt;
lsmeans  trt/pdiff stderr;
run;

proc GLM;
class animal trt;
model NHE2 = trt;
lsmeans  trt/pdiff stderr;
run;
```

```
proc GLM;
class animal trt;
model ATP1 = trt;
lsmeans  trt/pdiff stderr;
run;


proc GLM;
class animal trt;
model NHE1s = trt;
lsmeans  trt/pdiff stderr;
run;


quit;
```

Analyze volatile fatty acid distribution data.

```
Data Fistulated Steers;
input steer time trt Acetate Propionate Isobutyrate Butyrate Isovalerate Valerate Ratio Total;
time1=time;
Datalines;
;


title 'Acetate';
proc mixed;
class steer trt time;
model acetate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;


title 'Propionate';
proc mixed;
class steer trt time;
model propionate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;


title 'Isobutyrate';
proc mixed;
class steer trt time;
model isobutyrate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;


title 'Butyrate';
proc mixed;
class steer trt time;
model butyrate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;
```

```
title 'Isovalerate';
proc mixed;
class steer trt time;
model isovalerate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;


title 'Valerate';
proc mixed;
class steer trt time;
model valerate = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;


title 'Ratio';
proc mixed;
class steer trt time;
model ratio = trt time trt*time;
random steer;
repeated time/type=sp(pow)(time1) subject=steer rcorr;
lsmeans trt time trt*time/pdiff;
run;
```