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## Estimating Dry Matter Digestibility of Forage in Equine Diets

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# **ESTIMATING DRY MATTER DIGESTIBILITY OF FORAGE IN EQUINE DIETS**

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Taylor N. Godwin

December 2019

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# ESTIMATING DRY MATTER DIGESTIBILITY OF FORAGE IN EQUINE DIETS

Agriculture

Missouri State University, December 2019

Master of Science

Taylor Nichole Godwin

## ABSTRACT

Previous research indicates equine fecal inoculates produce comparable results to cecal fluid when used for *in vitro* procedures to analyze dry matter digestibility (DMD). Equine hindgut microbial populations represented in fecal samples have been shown to be affected by diet. The objective of this study was to determine the effect of diet on *in vitro* DMD when fecal samples from horses on varying starch and fiber diets were used as inoculates. Six mature Quarter Horses (BW  $522 \pm 45$ kg) were used in a crossover repeated measures design to compare the effects of a grain vs. all forage diets on the *in vitro* digestion of forages ranging: CP 7.7 to 16.4 %DM, NDF 53 to 72 %DM. Treatments were a grain diet of corn chops and mixed grass hay with alfalfa cubes and a forage only diet of mixed grass hay with alfalfa cubes. Horses were divided into two groups of three and assigned an initial start diet. Fecal samples were collected from each horse on days 0, 11, 22, 33, and 44, with groups switching treatments on day 22. Inoculum prepared from samples were used in the Ankom Daisy II Incubator® to compare DMD after fermentation. Digested NDF (NDFD) and digested ADF (ADFD) of forage samples were analyzed using the Ankom<sup>2000</sup> Fiber Analyzer®. Data were analyzed using the MIXED procedure of SAS using covtest to evaluate digestibility differences in the diet by forage parameter interaction. After the 22d adaptation period, fecal samples from horses on the grain diet promoted higher NDFD of forages with high NDF and low CP when CP and NDF were used as covariates ( $P=0.04$  and  $0.03$ , respectively). Dry matter digestibility tended to be higher ( $P=0.08$ ). There was a significant horse effect on DMD and NDFD ( $P \leq 0.05$ ). Findings suggest diet may influence hindgut microbial population to the extent of effecting fiber digestion and should be considered when selecting equine fecal samples for *in-vitro* digestion procedures. Increased digestibility of low-quality forage was seen when horses were fed a grain diet. In addition, pooling fecal samples from more than one donor consuming various diets may yield more precise results for *in vitro* digestibility trials. Further investigation of forage digestibility using a broader spectrum of forage types may elucidate a more distinct pattern of the dietary impact on fiber digestion.

**KEYWORDS:** Equine, microbial population, fermentation, digestion, NDF, ADF

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A Master's Thesis  
Submitted to the Graduate College  
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December 2019

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

## ACKNOWLEDGEMENTS

I can't believe the time has already come for me to write an acknowledgments page! I would like to thank those who have played an integral role in, not only this project, but teaching, supporting, and at times, challenging me over the course of my graduate studies. Primarily I would like to thank my advisor, Dr. Gary Webb, and committee members Dr. Walker and Dr. Sukovaty for the opportunities that taught me far beyond the confines of a classroom. Webb, I really appreciate- are you still reading this? Presence of the previous sentence in the final draft indicates he indeed, was not. I would also like to express my appreciation to Dr. Philip Lancaster for his vital role in this project's statistical analysis and interpretation of results.

I would like to thank course instructors, Will Boyer and Sue Webb for your stories, advice, and help with logistics during and after the research trial. And, of course, my fellow graduate students Jordan Shore and Delaney O'Donnell for your friendship and humor during this challenging process. Finally, I need to thank my friends and family for supporting me and bringing uplifting words of wisdom when it seemed the sky was falling!

To all of those listed above, you have made my time here at Missouri State University unforgettable. Whether it be fate or divine intervention that guided me to this graduate position, I wouldn't trade the friends, mentors, or experiences made for anything!

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## INTRODUCTION

### Justification of the Study

According to the American Horse Council Foundation (AHCF), the total economic impact of the equine (*Equus caballus*) industry has increased by 20 percent from 2005 to 2017. More than 1.7 million people account for \$79 billion in salaries, wages, and benefits associated with the 7.2 million horses in the United States (United States Department of Agriculture, 2017). Thirty-eight percent of horses are used in physically demanding ventures while 8.5 percent are used for breeding purposes accounting for nearly half of the equine population in the United States (United States Department of Agriculture, 2017). Physically demanding pursuits require a high level of dietary energy intake, commonly accomplished with the addition of cereal grain. However, this addition is not without consequence as increased cereal grain in equine diets has reportedly caused gastric disruption. Horses are highly susceptible to digestive upset, disruption of the microbial community within the hindgut resulting from digestive upset can have fatal consequences such as laminitis and colic (Milinovich et al., 2006; Harlow et al., 2014)

For equine to perform at the desired level, a diet that meets the nutritional requirements should be fed. Dry matter digestibility (DMD) is currently used to estimate the value of forage in the equine diet. Dry matter digestibility is especially important when assessing the nutrient content of feedstuff (Hansen and Lawrence, 2017). Knowing the DMD of the forage allows for a more accurate estimate of the availability of nutrients for digestion and assigning a value accordingly. Traditionally, chemical evaluations, in vitro digestive systems, and near infrared reflectance spectroscopy have been used to estimate the value of feedstuff (National Research Council, 2007).

Methods have been developed to assist with evaluating forage quality using the results obtained from lab analysis. Van Soest et al. (1991) pioneered the first endeavor to partition dry matter into fractions according to bioactivity in the rumen. Neutral detergent fiber and acid detergent fiber (NDF and ADF) are commonly used when determining the digestibility and intake of forage. According to Jeranyama (2004), relative feed value (RFV) has been used for years to compare the quality of legumes, legume/grass hay, and silages in ruminants. To calculate RFV, the forage must be analyzed for NDF and ADF then DMD and dry matter intake (DMI) can be estimated using their relationship to ADF and NDF, respectively. Finally, DMD is multiplied by dry matter intake (DMI) and divided by 1.29 (Jeranyama, 2004). In the past, the RFV has been trusted to price forage, however, relative forage quality (RFQ) has demonstrated to predict an animal's performance more accurately when fed the forage in question (Jeranyama, 2004). Unfortunately, calculating RFQ requires extensive nutrient analysis, to which a more widely used analysis such as crude protein (CP), NDF, and ADF is preferred. While the reported NDF and ADF estimates derived from laboratory analysis have been relatable to ruminants, it is unclear how they are associated with the digestion by the equine digestive tract (Hansen and Lawrence, 2017).

Volatile fatty acids (VFAs) produced as a result of bacterial fermentation of feedstuff in the equine hindgut accounts for as much as 50 percent of the horses daily digestible energy requirements (Vermorel and Martin-Rosset, 1997). Previous research has characterized the diversity in the equine cecal microbiome yet failed to produce a defined core bacterial population of a normal healthy equine GIT (Ericsson et al., 2016). Differences in management practices, age, genetics, diet, and body condition of horses affect the composition of the hindgut microbiome (Steelman et al., 2012). As substrates become available to the microbial population

in the hindgut, microbes, occupying specific niches, fluctuate depending on their capacity utilize available substrates as energy sources and withstand metabolites resulting from fermentation (Kristoffersen et al., 2016). Because of the microbial population changes in the hindgut, forages may vary in digestibility when concentrate is added to the individual's diet.

### **Need for Study**

Second to in vivo digestibility trials, in vitro studies are considerably reliable when estimating DMD of forage in an equine diet (Lowman et al., 1999). Unfortunately, as digestive trials are labor intensive, time sensitive, and require a cecal fluid or fecal inoculate, it is neither efficient nor practical to assess every forage. Differences in digestibility among individuals have been observed (Dougal et al., 2014). Therefore, in vitro studies largely depend on the source of the inoculum. Forages are commonly analyzed for NDF and CP and yield quick results in comparison to the 48h in vitro trial. An accurate estimation of DMD provides a prediction of digestibility for the average horse allowing for proper diet formulation to meet the individual's requirements.

Dense in energy, cereal grains are typically added to the ration of performance horses. However, bypass starch reaching the hindgut is rapidly fermented, resulting in the proliferation of amylolytic bacteria and accumulation of lactate, which decreases hindgut pH (Julliand et al., 2001; Harlow et al., 2014). Decreased numbers of cellulolytic bacteria have also been observed when horses are fed a high starch diet (Julliand et al., 2001). An acidic pH in the lumen of the hindgut has been implicated as the cause of ecological disruption of microbes, laminitis, and colic in horses (Harlow et al., 2014). Yet, it is unclear if the dominant microbial population shift from cellulolytic to amylolytic causes a decrease in forage digestibility. Therefore, research

should be conducted to determine availability of nutrients using forage composition, thus, allowing horses to operate efficiently for the desired purpose while minimizing the costs associated with overfeeding.

## **Objectives**

The purpose of this study was to evaluate the forage parameters and the effect of fecal donor diet on the digestibility of NDF, ADF, and DM. The specific objectives of the study were to (1) expand the database of digestibility trials, (2) determine the difference in digestibility of neutral detergent fiber, acid detergent fiber, and dry matter when horses are fed a forage only diet and a high grain: forage diet, and (3) determine if NDF, ADF, and CP could be used to predict fiber digestibility.

## **Null Hypotheses**

Null hypothesis of the present study was that diet of fecal sample donor would not affect digestibility of dry matter, neutral detergent fiber, or acid detergent fiber. In addition, forage parameters such as crude protein, neutral detergent fiber, and acid detergent fiber could not be used to estimate digestibility of fiber.

## LITERATURE REVIEW

### Equine Gastrointestinal Anatomy

In its entirety, the equine GIT is comprised of the mouth, esophagus, stomach, small intestine, cecum, colon, and rectum. Further, the small intestine is distinguished into the duodenum, jejunum, and ileum and the colon into ascending (large) and descending (small) regions (Krunkosky et al., 2017). Segments of the equine digestive tract are grouped according to their function and common purpose; thus, the mouth, esophagus, stomach, and small intestine are referred to as the foregut while the cecum, colon, and rectum are known as the hindgut.

Once in the mouth, feedstuff is masticated with the addition of salivary  $\alpha$ -amylase and swallowed to travel down the esophagus and gain entry to the stomach via the muscular cardiac sphincter (Krunkosky et al., 2017). The oblique angle at which the esophagus meets the cardia of the stomach in combination with the striated musculature of the cardiac sphincter contributes to the horse's inability to regurgitate even through gastric disruption when regurgitation would be beneficial (Krunkosky et al., 2017).

Connected to the abdominal wall and neighboring organs by the dorsal and ventral mesogastria, the stomach resides with the cardia adjacent to the diaphragm. The most cranial regions of the stomach, the cardia and fundus contain non-glandular squamous epithelial cells expressing acid sensitivity similar to the esophagus (Krunkosky et al., 2017). Following the greater curvature of the stomach leads to the margo plicatus, a thick fold of cells serving as a transition from the non-glandular stratified squamous cells to the simple columnar cells within the glandular body and pylorus of the stomach. Consequently, it is here that gastric ulcers are commonly found as non-keratinized epithelial cells located near the margo plicatus are predisposed to frequent exposure to gastric acids.

In the stomach, three glandular regions exist (proper gastric, cardiac, and pyloric) together, these glands account for gastric secretions including hydrochloric acid (HCl), pepsinogen, histamines, somatostatins, gastrin, and a bicarbonate-rich mucus to protect the surface epithelium (Krunkosky et al., 2017). Secretion of HCl is continuous, resulting in a highly acidic environment ( $\text{pH} \leq 2.0$ ) in a fasting state attenuated only by the prehension of a meal that absorbs gastric acid and stimulates secretion bicarbonate-rich saliva (National Research Council, 2007).

Compared to the total capacity of the equine GIT, the stomach is small. However, digesta may remain in the stomach for up to 2-6h before transitioning to the duodenum (National Research Council, 2007). Digesta entering the stomach initiates peristaltic contractions, inevitably exiling old digesta to the duodenum of the small intestine through the pyloric sphincter of the stomach. Further distention of the stomach increases digesta expulsion and contractions that persist through the small intestine, successfully dictating the rate of passage to the cecum (Van Weyenberg et al., 2006).

When stretched, the average mature equine's small intestine extends 26.5m in length; however, segments of the small intestine (duodenum, jejunum, and ileum) are partitioned based on function rather than equal thirds. Ventral to the liver, the duodenum is approximately one meter in length and directly accessed by pancreas and liver secretions through the pancreatic and bile ducts, respectively (Krunkosky et al., 2017). An abundance of villi coated with microvilli are present on the luminal side of the duodenum. Microvilli, also known as the brush border, are covered with hair-like projections called glycocalyx and glazed with viscous aqueous mucus secreted from Brunner's glands (Blikslager, 2017). Collectively, villi and related components provide surface area for feed particle to be exposed to the digestive enzymes secreted by the

brush border (Blikslager, 2017). Acidic conditions, caused by hydrogen ions in the lumen, stimulate S cells to release secretin, subsequently resulting in the secretion of bicarbonate from the pancreas to prevent mucosal damage. Further, brush border enzymes operate optimally at a nearly neutral pH. Although basal pancreatic excretions are continuous (10-12L/100kg body weight (BW)/day), presence of fat and protein in the duodenum stimulates cholecystokinin from villi endocrine cells which in turn, triggers the release of enzymes and cofactors for carbohydrate (CHO), fat, and protein digestion (Blikslager, 2017). Villi, previously long and thin to increase surface area and, therefore, uptake of nutrients from digesta, take on a short club shape when transitioning to the ileum from the jejunum. Enzymatic digestion and absorption continue in the longest segment of the small intestine, the jejunum (25m); however, the absorptive capacity decreases as digesta migrates towards the ileum (0.5m).

Present throughout the small intestine, crypts of Lieberkühn secrete extracellular fluid and immunoglobulin A (IgA) to maintain fluidity of chyme and clear crypts of potentially infectious agents (Blikslager, 2017). Larger molecules such as D-glucose and D-fructose require transporters into the cellular matrix like SGLT-1, a sodium dependent transporter specific to the D-isomer of glucose; yet, minerals and water can pass paracellularly or use transporters for transcellular diffusion (Blikslager, 2017). Presence of transporters also decreases in frequency approaching the terminal end of the small intestine; this, in combination with the differential shape and number of villi, accounts for the attenuating absorptive capacity as digesta approaches the ileo-cecal valve.

Expulsion from the ileo-cecal valve marks the transition of digesta escaping enzymatic and chemical digestion in the foregut to microbial fermentation in the hindgut. The cecum, colon, rectum, and anus are collectively represented by the term hindgut. Reportedly, the transit



time to the cecum is approximately 2h post ingestion (Argenzio et al., 1974). It has been demonstrated that rate of passage is reduced in the hindgut to allow time for fermentation as unabsorbed particles spend 75 to 85 percent of total mean retention time (23-48 h) here (Van Weyenberg et al., 2006).

Microorganisms in the hindgut and the host have symbiotic relationship that may turn pathogenic under certain circumstances. Ideally, the large intestine provides an accommodating environment to resident bacteria capable of fermenting fibrous plant matter and in turn, produces volatile fatty acids (VFAs) used in host metabolism. High mobility, generous size, distinct differences in diameter, and intricate functions of the large intestine lead to the predisposition of potentially fatal digestive upset (Argenzio et al., 1977).

A fermentation vat analogous to that in ruminants is found in the first region of the hindgut known as the cecum. Entrance to the cecum via the ileo-cecal valve prevents retrograde flow of digesta to the small intestine (Argenzio et al., 1974). Up to 30L of undigested feedstuff spends an average of 8h in the 1m long cecum. Structurally, the cecum is composed of three regions (base, body, and apex) containing sacculations (haustra ceci) and arteries, veins, and lymph vessels through the mesentery for circulation and absorption. Coordinated muscular contractions in the cecum encourage mixing of digesta with microbial populations found in various regions and contact with mucosa for absorption. Initiating in the body, proximal to the apex, retrograde propagation moves toward the base using the slightly negative pressure (in relation to atmospheric pressure) to move ingesta and gas. Contractions continue to constrict, forming two demarcated regions causing the cecal base to rise and permitting passage through the cecocolic ostium once the cranial region of the base is contracted (Nieto and Rakestraw,

2017). Though contractions persist every two minutes, most digesta remains in the cecum to continue fermentation.

Like the cecum, the colon exhibits haustra-to-haustra mixing movements with peristaltic contractions moving digesta aborally. The capacious ascending colon is 25cm in diameter and may contain as much as 80L of ingesta. In addition, the ascending colon lacks proper attachment to the abdominal wall leaving the potential for displacement (Lopes and Johnson, 2017).

Progression to the descending colon, representing 3-4m of the distal large intestine, the pelvic flexure narrows to an 8cm diameter. Subsequently, digesta enters the rectum (25cm length) before exiting through the anus. A marked decrease in diameter from the ascending and descending colon elicits a high retention time in the large colon. Digesta passage through the pelvic flexure can be impeded through compaction, in the case of impaction colic, this region is often implicated.

Because of the large intestine's bidirectional capacity to absorb and secrete water, it is speculated to be a stored reserve of water, readily used when the animal is in a dehydrated state (Meyer et al., 1995). As a response to the horses physiological state, aquaporins are inserted in the membrane of colonocytes, used to passively channel water through the luminal and basement membranes in order to keep oncotic, osmotic, and hydrostatic pressure in balance (National Research Council, 2007). Feeding a roughage only diet results in one and half times the water found in the large intestine of horses fed a concentrate mixed ration Meyer and colleagues (1995).

As an anaerobic environment, the large intestine provides optimal conditions for microbial fermentation; however, activity of the microflora is a result of substrate availability (National Research Council, 2007). In ruminants, microbial fermentation of intake protein occurs

prior to the host's enzymatic digestion in the small intestine leading to efficient absorption of microbial crude protein produced in the rumen. Unfortunately, as horses are hindgut fermenters, microbial crude protein is poorly absorbed and therefore excreted in feces. However, non-protein nitrogen is passed through the intestinal mucosa supplying excessive ammonia to be excreted in the urine. As a potential benefit, a healthy hindgut microflora binds nitrogen in the process of constructing microbial crude protein, therefore, preventing its absorption and allowing passage to feces (Nieto and Rakestraw, 2017).

### **Fermentation Products**

A strict herbivore, the horse has evolved a gastrointestinal tract (GIT) capable of adapting to environmentally available nutrients rich in fibrous plant material. To aid in the degradation of plant structural carbohydrates, horses evolved an alternative to the foregut fermentation vat found in ruminants. Because they have a simple glandular stomach, horses are considered a monogastric species having a large functioning cecum with a resident microflora proficient in extracting short chain fatty acids (SCFA) from feedstuff through fermentation. Short chain fatty acids are absorbed through cecal or large intestine mucosa then transported through the bloodstream to the liver to make energy for the host (Vermorel and Martin-Rosset, 1997). Nutrients escaping enzymatic and chemical digestion and subsequent absorption enter the most proximal segment the digestive tract, collectively known as the hindgut. Here, with the aid of symbiotic microorganisms, nutrients which escaped mammalian digestion is fermented, producing SCFA for host and neighboring microbe metabolism.

Although nonstructural carbohydrates are primarily digested enzymatically in the small intestine, VFA production from microbial fermentation accounts for more than 50 percent of

daily energy requirements in horses (Vermorel and Martin-Rosset, 1997; Shepherd et al., 2012). In contrast, ruminants such as sheep, goats, and cattle are foregut fermenters and receive in excess of 70 percent of daily energy requirements from VFAs. It has been shown that horses on a mixed grain and forage diet attain 25 percent of their energy from the degradation of feedstuff through the small intestine while more than 50 percent was through the production of energy using absorbed VFAs (Lopes and Johnson, 2017). Ruminants have a compartmentalized stomach allowing for regurgitation, remastication, and rumination, a process that permits time for the microbial population to access feed particles for digestion. Though horses are unable to ruminate, and location of fermentation differs, the resulting products are similar to a ruminant. Volatile fatty acids consist of the two, three, and four carbon molecules, acetate, propionate, and butyrate (Hintz et al., 1971). In beef cattle a low acetate: propionate + butyrate ratio is desired to increased energy efficiency through propionate hepatic glucose synthesis and to minimize methane production (Russell and Baldwin, 1979).

In the horse, monocarboxylate transporters (MCT) are responsible for the uptake of VFAs at a rate inversely proportional to molecular weight, made apparent by their presence in the large intestine correlating to the concentration of VFAs absorbed (Argenzio and Southworth, 1975). Compared to cattle, absorption through the large intestinal and rumen epithelial tissues are remarkably similar when evaluated on the basis of dry tissue weight (Argenzio et al., 1977). Imperative to this exchange, a proton can enter the rumen or large intestinal epithelium by exchange with sodium or acting as a buffer in a molecule of bicarbonate. Volatile fatty acids are mostly (>99 percent) protonated when transported through the cell, which requires a luminal pH higher than the average pka of the VFAs (4.8) and a hydrogen ion source (Argenzio and Southworth, 1975).

When fed a high starch diet, containing approximately 30 percent starch, a shift is created in the hindgut microbial population inherently causing an increase in the molar concentration of propionate with a concurrent decrease in acetate (Drogoul et al., 2001). Propionate is thought to be the most important VFA in terms of glucose regulation and homeostasis in forage fed horses . For every two molecules of propionate absorbed and delivered to the liver via the portal vein, one molecule of glucose can be made through gluconeogenesis (Argenzio and Hintz, 1970). However, acetate, the principle VFA produced in forage fed horses, can be used aerobically for energy immediately following uptake. Pethick et al. (1993) observed 30 percent of energy used by hindlimbs was attained through use of acetate. Circulating acetate exceeding the present energy requirements is stored in peripheral tissues as long-chain fatty acids (National Research Council, 2007). However, research has revealed another potential purpose for these short chain fatty acids.

Using mice as a model, Cornall et al. (2011) and Shepherd et al. (2014), validated that short chain fatty acids (mostly acetate) bind to G-coupled protein receptor 43 (GPR43) as a ligand regulating metabolism. According to (Ge et al., 2008), the attachment of acetate or propionate attenuates lipolytic lipolysis resulting in the suppression of plasma free fatty acids. Additionally, GPR43 is prominently found in monocytes, macrophages, and neutrophils (Brestoff and Artis, 2013). When bound, neutrophilic function is increased suggesting VFAs may place a vital role in the immune system. A deficit in VFAs results in autophagy linked to immunity and the integrity of the intestinal barrier (Patel and Stappenbeck, 2013). Thus, if a VFA deficiency leads to the loss of a proper intestinal barrier, it could also be associated with the permeable intestinal barrier seen as a result of hindgut acidosis.

## Feed Management

Management practices, such as diet and feeding schedules, are key contributors to the onset of the non-specific abdominal pain, commonly referred to as colic, though there are many potential causes. Previous reports indicate the incidence of colic ranges from 0.9 to 10 episodes per 100 horses in a given year (Cohen, 2017). Interestingly, the risk of colic is significantly elevated in horses that have previous experience with the disease. The incidence increased to 35 episodes per 100 horses in a year (Scantlebury et al., 2011). However, colic is not the only disease related to metabolic dysfunction. Colic, in conjunction with equine metabolic syndrome and laminitis, provides additional concern for horse owners, warranting an investigation of the equine GIT and suitable diets.

**Structural Carbohydrates.** Structural carbohydrates within the plant cell wall, cellulose, hemicellulose, pectin, and lignin, contribute to the fiber component of rations and have been partitioned by Van Soest et al. (1991) according to their availability in the rumen. Neutral detergent fiber (NDF) consists of hemicellulose, cellulose, and lignin, while acid detergent fiber (ADF) is composed of only cellulose and lignin. Cellulose, hemicellulose, and pectin are molecules of carbohydrates linked by  $\beta$  1-4 glycosidic bonds; however, cellulose contains solely glucose, hemicellulose is arabinose, xylose, glucose, fructose, mannose, and galactose molecules, and pectin polymers are molecules of arabinose, galactose, and mannose. Although not a carbohydrate, lignin is an indigestible, undefined compound from the phenylpropanoid pathway that aids in the lignification of cellulose contributing to the plant's rigidity, increasing as the plant matures (Hartley and Jones, 1977). Mammals possess enzymes to digest  $\alpha$  1-4 and  $\alpha$  1-6 glycosidic linkages, however,  $\beta$  bonds render structural carbohydrates indigestible by endogenous enzymes within the GIT. Microbial populations in the hindgut readily digest pectin

but take additional time to ferment cellulose and xylans (linked xylose residues from hemicellulose digestion) as they are stable polymers and more resistant to degradation (Keys et al., 1963). Although particles of lignin may be broken down during fermentation, it is considered the most indigestible portion of forage (National Research Council, 2007).

**Non-Structural Carbohydrates.** Conversely, cell contents are rich in plant protein, minerals, lipids, and NSC. The following are denoted as non-structural carbohydrates: simple sugars (mono- and di- saccharides), fructans, and starches (polymers of alpha 1-4 linked polymers of fructose and glucose, respectively). Cell contents are readily digestible and fermentable, contributing to the nutritive value of forage. Protein is largely found in the leaf portion of forages and thought to contribute to the evaluation of forage quality. Alfalfa and timothy leaves have demonstrated to contain 2 to 3-fold more protein than the stem (Jeranyama, 2004). Starch, the major storage carbohydrate in vegetative legumes and warm-season grasses, is produced and stored within cell chloroplast, therefore, limiting its production through saturation. Cool-season grasses produce fructans to store with starch as a minor contributor (Turner et al., 2006). However, unlike warm-season grasses and legumes that are self-limiting in terms of carbohydrate storage, cool-season grasses may accumulate considerable amounts of fructan as excess photosynthate is transported from the leaf to the stem. Therefore, more fructan is found in the stem than the leaf of cool-season grasses. In temperate cool-season grasses, starch has been reported to account for as little as 10-15 percent of carbohydrate storage, while fructans can reach up to 50 percent of dry matter (Longland and Cairns, 1998). As a result, cool-season grasses generally contain a greater concentration of NSC compared to warm-season grasses and legumes. In ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), fescue (*Festuca arundinacea*), and orchard (*Dactylis glomerata*) grasses, 1.5 to 10-fold more fructan was found

in the stem compared to the leaves of the same forage (Waite and Boyd, 1953). The aforementioned forages have the highest concentration of NSC in the late spring, declining mid-season, and rising to median levels at the start of autumn (Waite and Boyd, 1953). When fructan concentrations are high in the forage present in pastures grazed by horses, disruption of the normal hindgut microbiota can occur, causing the induction of pasture associated laminitis (Milinovich et al., 2006). Conditions that subdue photosynthetic activity and amplify plant growth results in lower concentrations of non-structural carbohydrates in plant vegetative tissue. Maturity affects the NSC found in forages, having a significant impact on leaf to stem ratio.

**Maturity.** Maturation of a plant results in the attenuation of leaf growth, stem elongation, development of reproductive structures such as flower and seed-heads, photosynthate accumulation and decrease in cell content: cell wall ratio (Givens et al., 1992; Dulphy et al., 1997). The intertwined relationship of lignin to cellulose, hemicellulose, and pectin renders the closely associated structural carbohydrates recalcitrant to digestion and ultimately decreases the nutritive value of forage (Hartley and Jones, 1977). Plant maturity is a valid consideration for forage quality, and digestibility as a decrease in leaf to stem ratio is exhibited along with a decrease in protein and increase in the plant's fiber component (Figure 1). Development of a seed-head or flower has been implemented as the critical point of maturity, marking the initiation of protein decline (Harkess and Alexander, 1969).

Ideally, a pasture tailored for grazing horses would consist of legume and mixed grasses to provide a source of dietary fiber, carbohydrates, and protein. Cool-season grasses are generally more digestible than warm-season as they contain a higher concentration of easily degraded cells high in NSC as compared to the high proportion of recalcitrant tissue of warm season grasses (Akin and Burdick, 1975). In terms of protein, legumes possess the highest



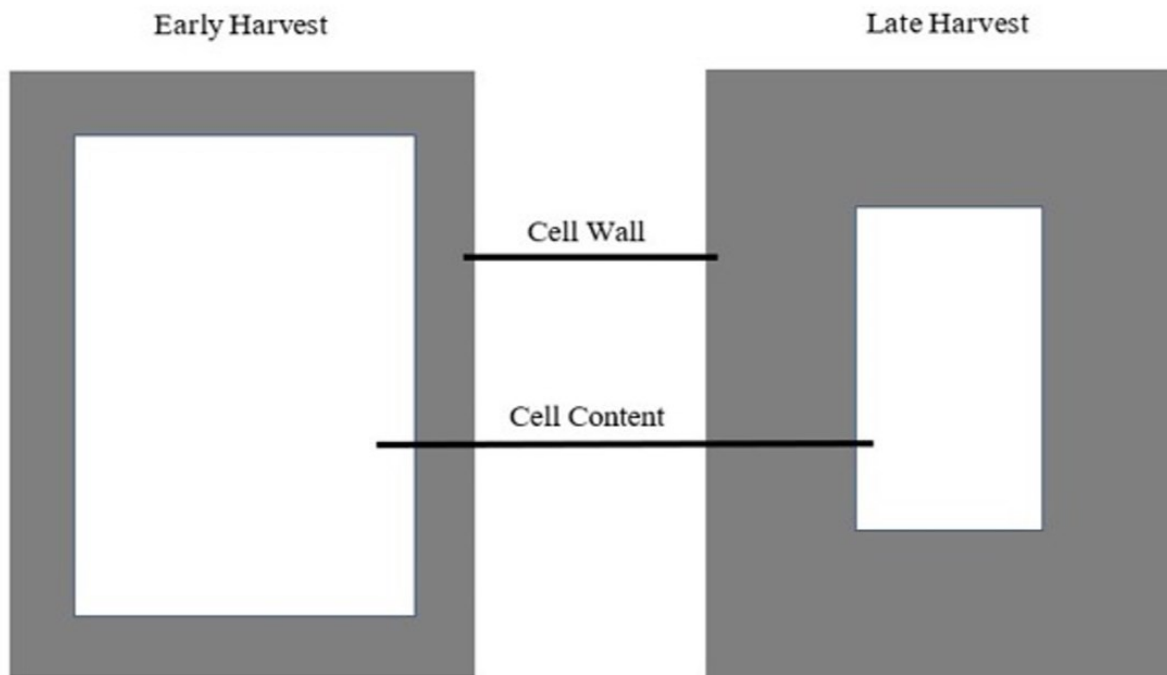


Figure 1. Differences in plant cell wall ratio due to maturity

quantity due to the ability to fix nitrogen, with cool-season, then warm-season grasses to follow. A further comparison to cool-season grasses reveals that legumes are lower in hemicellulose yet higher in pectin components. In addition, warm-season grasses mature more rapidly, resulting in a highly lignified cell wall decreasing digestibility (Waite and Boyd, 1953).

**Cereal grain.** Increasing equine activity requires the addition of an energy dense feedstuff. However, The National Research Council (2007) recommends no more than 0.2-0.4 percent body weight (BW) of starch. Depending upon the type of cereal grain, granule composition, and method of processing, starches are readily available and easily digested in the small intestine. However, overloading the digestive capacity of the foregut results in starch bypassing enzymatic digestion and entering the cecum. Bypass starch acts as a rapidly fermentable substrate for bacteria in the cecum.

Cereal grains are the most common source of starch used to increase energy concentration in domestic animals (Svihus et al., 2005). The endosperm of monocots contains the starch rich portion of the seed, which is encased in the pericarp or “bran”. The pericarp accounts for the fibrous component of seed. However, fiber from the pericarp is comparably low (approximately 10 percent NDF) in relation to forages. Within the endosperm of the cereal grain, starch granules are encapsulated by prolamins (zein), a protein matrix that serves as a protective mechanism effectively inhibiting enzymatic starch digestion. Starch is constructed of amylose and highly branched amylopectin, both containing  $\alpha$ 1-4 linked glucose molecules with  $\alpha$ 1-6 branching points (Svihus et al., 2005). Starch granules contain alternating semi-crystalline and amorphous layers of amylopectin and amylose and a lipid component. Increased lipid content, commonly seen in small starch granules, are negatively associated with digestibility as the hydrophobic lipids reduce contact to digestive enzymes and extent of swelling during processing (Svihus et al., 2005). A harder or vitreous endosperm is preferred as soft endosperm are loosely packed containing more proteins preventing damage to starch granules, ultimately rendering the advantages of processing an ineffective method of increasing digestibility (Svihus et al., 2005).

Processing affects the structure of starch granules disrupting the ability to isolate starch from the exterior. Kienzle et al. (1998) reported only 29 percent pre-ileal digestibility of whole or broken maize corn. However, this number was increased to 47 percent when starch granules were damaged by grinding and even further to 90 percent when gelatinization was used to completely destroy granules (Meyer et al., 1995). In the small intestine, corn is digested through a process called endocorrosion in which amylase gains access to starch through small pinholes in the zein protein matrix. Once inside, amylase releases glucose molecules through starch degradation (Kienzle et al., 1998). Starch passing the ileum is passed to the cecum where

cellulolytic and amylolytic bacteria concentrations vary with composition of substrates (Goodson et al., 1988; Julliand et al., 2001; Harlow et al., 2015).

## **Microbiome**

Though studies vary in compositional proportions, the equine hindgut has a residence of bacteria (71-99 percent), archaea (1-13 percent), and protozoa 14.5 percent (Shepherd et al., 2012; Fernandes et al., 2014). The neonate equine hindgut is innately sterile then inoculated through eating the feces of mature horses sharing a pasture (coprophagy) and bacteria present on their dam's teats when suckling (Crowell-Davis and Houpt, 1985). Protozoa and archaea have a limited role in fermenting cellulose but possess the ability to digest pectin (Julliand et al., 1999). Yet, when horses were abruptly changed to a high concentrate diet, protozoa decreased 48h after transition, while archaea remained at a constant abundance (Goodson et al., 1988; Fernandes et al., 2014). The progression back to a forage diet did not reestablish the previously measured protozoan numbers. Although little research has been done to identify the necessity of archaea and protozoa, extensive studies of bacteria have been performed with various methods and outcomes (Harlow et al., 2015).

Identification of a core microbial community in the human gastrointestinal tract has linked diversity, species, and increased species richness to obesity and metabolic dysfunction (Dougal et al., 2013). However, it is unknown whether the core community is driven by genetics or strictly function in horses. In addition, a core community's existence in the equine digestive tract has been called into question for the lack of permanency in research results (Turnbaugh and Gordon, 2009; Sekelja et al., 2011; Dougal et al., 2013).

Changes in the hindgut are rapid, and differences may be attributed to the microbiome's response to host diets or other intrinsic characteristics. Fernandes et al. (2014), have suggested the vast diversity of microflora in the hindgut is an evolutionary advantage as it contributes to the equine's adaptability to type and quality of forage seasonally available in the environment. Rich in fibrolytic inhabitants, hindgut fermentation allows herbivores to succeed on cellulolytic plant matter low in hydrolysable carbohydrates (Shepherd et al., 2012).

Throughout the literature, there are many contrasting results regarding the species present in the hindgut; it was suggested by (Costa and Weese, 2012), that these inconsistencies and missing evidence of composition and function of hindgut microflora can be attributed to the lack of continuity between experimental designs, sequencing methods, phylogenetic, and statistical analyses. In addition, culture-dependent studies have endured the short comings of enumerating anaerobic bacteria, an estimated 80 percent of the population in the hindgut, in laboratory settings leading to a misrepresentation of populations present in the hindgut (Kern et al., 1974). Technological advances have provided culture-independent methods, including: DNA pyrosequencing, the Sanger method, ion semiconductor sequencing, sequencing small subunit rRNA, and group classifications through DNA fingerprinting techniques. However, these methods unveiled additional gaps as the library used to identify sequences reveal a generous portion of bacteria lacking a previous description (approximately 89 percent of recovered species). When extracting microbial DNA from equine fecal samples, the PowerFecal<sup>®</sup> kit (QIAGEN<sup>®</sup>, Germantown, MD) has reportedly been the most accurate (Hart et al., 2015). Classifying microbes according to their functional importance in the hindgut presents a further challenge as there are sure to be functional redundancies in that varied species are accomplishing similar tasks (Turnbaugh and Gordon, 2009).

As an initial step in classifying bacteria, a Gram stain is commonly performed to determine easily distinguished subjective characteristics and morphology. In a Gram stain, a bacterial colony is smeared and fixed to a glass slide and drops of crystal violet cover the smear. After one minute, the slide is washed, and iodine is added to form a complex with the crystal violet, locking it within the peptidoglycan layers of gram-positive bacteria. Following iodine, two drops of decolorizer are added stripping the complex from gram-negative bacteria, and a counter-stain, safranin, is added prior to another wash and viewing it under a 1000x oil emersion lens. Bacteria that appear pink or red are considered gram negative (G-) while those appearing deep purple are gram positive (G+). As exogenous chemicals first contact the cell's outermost membrane, Gram staining provides valuable information regarding membrane characteristics (Flythe and Aiken, 2010).

The population of the equine GIT has been characterized at a phylum level by several studies (Dougal et al., 2013; Dougal et al., 2014; Venable et al., 2017), most of which name Firmicutes the predominate phylum across all horses. The phylum Bacteroidetes is most frequently referred to as the second most abundant with proteobacteria to follow. When properly enumerated, bacteria classified in the Firmicutes phylum are often identified using a genome library. Unfortunately, due to the plasticity of the genome of bacteria in the Bacteroidetes phylum, short amplicon sequences, culturing techniques, and the limited Bacteroidetes genomic library, such identifications in the equine hindgut are rarely made. Thus, composition beyond the family level is not well defined in the equine hindgut (O' Donnell et al., 2017). Primarily, *Bacteroides* spp. and *Prevotella* spp. are the key representative members of the Bacteroidetes phylum in the GIT of humans, bovine, and equine (Daly et al., 2012).

Controversy exists regarding the ratio of Firmicutes and Bacteroidetes in a healthy equine GIT as it has been reported between 1:1 and 4:1 (Dougal et al., 2014; Harlow et al., 2015), respectively. Studies performed on mice have demonstrated the high energy harvesting capacity of a microbiome collected from obese mice used to inoculate the GIT of mice with a sterile gut or a lean phenotype (Turnbaugh et al., 2006). After gut inoculation, lean mice demonstrated increased weight gain and adiposity, with the microbial population having higher Firmicutes to Bacteroidetes ratio. Yet it remains unclear how obesity and insulin dysregulation impact the gastrointestinal microflora of the horse. In a study by Morrison et al. (2018) obese horses exhibited demarcated increases in the phyla Bacteroidetes, Firmicutes, and *Actinobacteria* spp. In contrast, Biddle et al. (2018) observed a decrease in the mentioned phyla in obese horses and Shepherd et al. (2014) found significant differences in biochemical markers but no difference in total bacteria, Firmicutes or Bacteroidetes. Results of these studies render the effect of age minute compared to the previous findings however they conclude that horses older than 19 years display a decrease in bacterial diversity within the hindgut with an overabundance of proteobacteria. Originally thought to have only a saccharolytic metabolism, *Bacteroides* and *Prevotella* spp. have been identified as having a large regulated genome with the capacity to switch substrates pending environmental availability (Thomas et al., 2011). Given the apparent adaptability of Bacteroidetes, an increase in abundance would be expected as a representation of a highly competitive ecosystem in the hindgut (Daly et al., 2012). The *Lachnospiraceae* family remains a stable component of the equine hindgut regardless of diet, age, and body condition as it contains cellulolytic and saccharolytic members (Willing et al., 2009; Steelman et al., 2012).

Classification at the phylum level does not reveal specific information regarding the ecosystem in the hindgut. Beyond the phylum classification lies differences in substrate

preference, pH tolerance, fermentation products, morphology, oxygen tolerance, and presence/absence of a cellular envelope and/or peptidoglycan cell wall.

**Cellulolytic Microorganisms.** Essential to metabolism, symbiotic bacteria residing in the hindgut of horses are primarily involved in the fermentation of fiber (Dougal et al., 2012). When formulating a ration, it is important to consider that horses are grazing herbivores and to maintain the integrity of digestive function and horse welfare, a diet with adequate fiber, as previously outlined, should be fed. Diets dense in fiber give rise to a more stable microbiome with decreased populations of bacteria associated with metabolic dysfunction (Ericsson et al., 2016). The dominant phyla represented in the cellulolytic guild are Firmicutes and Fibrobacter. However, research has revealed fibrolytic activity associated with species in the phylum Bacteroidetes, which has stimulated more interest in these highly adaptive bacteria (Thomas et al., 2011).

Numbers of cellulolytic bacteria are six times higher in the cecum than the colon, whereas amylolytic bacteria are represented in higher proportions in the colon (de Fombelle et al., 2003). This may be due to the association between rapidly fermentable carbohydrates and a more fluid digesta resulting in short retention time in the cecum. Conversely, a fibrous meal lingers within the cecum for greater retention time for fermentation by cellulolytic bacteria. In support, Simmons and Ford (1991), recorded 56 percent of blood glucose was synthesized from propionate as a result of fermentation in horses fed a forage only diet. Although acetate is produced in higher quantities in a hay diet, propionate predominantly contributes to blood glucose levels (Argenzio et al., 1974).

Presently, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* are recognized as the predominant cellulolytic species representing 4-12 percent of the population in the hindgut

(Julliand et al., 1999; Suen et al., 2011; Daly et al., 2012; Shepherd et al., 2014). These strictly anaerobic bacteria are acid-intolerant expressing a depression in growth and cellulose digestion as pH falls below 6.0 (Miwa et al., 1997). Unfortunately, an abrupt transition to a ration high in rapidly fermentable carbohydrates elicits lactate production exceeding the utilization in LUB, inevitably declining pH causing cellulolytic metabolism to desist.

Bacteria in the *Ruminococcus* family are G<sup>+</sup> cocci members of the phylum Firmicutes and Clostridial cluster IV and produce a unique cellulosome-enzyme complex to aid in the digestion of fiber. The cellulosome-enzyme complex has been shown in *R. flavefaciens* specifically and characterized by as many as five active sites to degrade cellulose and xylans within hemicellulose. Cellulosomal xylanases are upregulated by more than 50-fold in the presence of cellulose and grows in the fibrous portion of feces where its preferential substrate is dense (Julliand et al., 1999). *Bacteroides* spp. in the phylum Bacteroidetes are anaerobic, G<sup>-</sup> bacillus capable of producing enzymes for the degradation of polysaccharides resistant to mammalian enzymes including cellulose, pectin, and xylan (Thomas et al., 2011; Flint et al., 2012). These bacteria utilize complex binding proteins to procure the substrate into the periplasm of the cell prior to degradation, a mechanism thought to preserve the substrate for their own catabolism, providing a competitive edge (Dodd et al., 2011). *Bacteroides* spp. have a large genome encoding enzymes for fiber digestion regulated with operons by substrate presence. *Bacteroides* spp. are capable of undergoing horizontal gene transfer to acquire catabolic pathways by inserting the genetic material from other bacteria into their own genome. Due to the plasticity of their genome, these bacteria adapt quickly to metabolize the substrates immediately available in the environment as an evolutionary advantage. Even more impressively, horizontal gene transfer is not limited by a lack of bacterial relatedness and contact with environmental bacteria



associated with food can also lead to its induction accounting for approximately 5.5 percent of the genome (Thomas et al., 2011; Flint et al., 2012). As a result, these non-adherent species are speculated to outcompete G<sup>+</sup> bacteria for the utilization of hydrolysable carbohydrates, yet the rate of degradation of fiber is unknown (Biddle et al., 2013).

*Fibrobacter succinogenes*, a member of the Fibrobacter phylum, operates in a unique fashion in relation to other fibrolytic bacteria. *Fibrobacter succinogenes* is a G<sup>-</sup> bacterium that expresses genes to hydrolyze hemicellulose; however, they lack the capacity to use xylans as a metabolic substrate (Suen et al., 2011). Further, they must attach to cellulose in order to ferment, yet they lack dockerins to facilitate adhesion (Kobayashi et al., 2008). The peculiarities do not stop here as *F. succinogenes* also accumulates copious amounts of glycogen representing between 60 and 70 percent of their dry cell mass (Morrison et al., 2018). A glycocalyx coats the outer membrane of the cell enabling the adhesion to the substrate and contact with enzymes present on the exterior (Miron et al., 2001). Though *F. succinogenes* will not excrete cellulases in the environment, they have shown to produce at least seven enzymes such as glucanases, cellodextrinases, cellobiosidase, and a cellobiase and cellobiose-phosphorylase (Béra-Maillet et al., 2004). Hemicellulose is degraded to allow access to cellulose, freeing xylans to contribute to the metabolism of surrounding bacteria.

**Amylolytic Microorganisms.** Starch utilizing bacteria such as *Lactobacillus spp.*, *Streptococcus spp.*, *Enterococcus spp.*, and some *Lachnospiraceae spp.* produce primarily lactate as a result of fermentation leading to a decrease in pH from hydrogen transfers. The introduction of non-structural carbohydrates to the hindgut provokes amylolytic bacteria to fervently proliferate, increasing the total number of bacteria and subsequent death of G<sup>-</sup> bacteria releasing endotoxins (Julliand et al., 2001; Bailey et al., 2003; Harlow et al., 2016). A balanced microflora

ecosystem relies on the symbiotic relationship between lactate producing and lactate utilizing bacteria (LUB) to maintain a pH optimal (6-7) to the inhabitants (Harlow et al., 2016).

*Veillonella* spp. are a group of G- bacteria implicated in catabolizing lactate and, in turn, producing acetate and propionate used in host metabolism. The aforementioned anaerobic bacteria are commonly found in the hindgut of horses and often considered members of the core microbiome. The group *Lachnospiraceae* contains both saccharolytic and fibrolytic microbes capable of degrading starch and hemicellulose and reportedly produce substantial amounts of the VFA butyrate (Daly et al., 2012; Dougal et al., 2013).

**Ecosystem.** Commensal and mutualistic relationships between hindgut microbes are imperative to maintain a healthy host. As previously outlined, groups of bacteria are capable of fulfilling metabolic niches presented in the hindgut and subsequently release metabolites that serve as substrates for neighboring bacteria. When fed a high fiber diet, bacteria with the fibrolytic guild will break down cellulose molecules, inevitably freeing some glucose molecules to the environment. Key amylolytic bacteria do not possess the enzymes necessary for SCHO digestion but utilize carbohydrates released from cellulolytic enzymes (Daly et al., 2012). This substantiates the continued colonization of amylolytic bacteria through a diet low in starch. Further Kristoffersen et al. (2016) mentions that *Treponema* spp. is a promoter of *F. succinogenes* activity aiding in its attachment to fiber in order to use discarded xylans as a substrate.

Krebs (1999), stated that in a climax community, multiple competitors cannot exist concurrently. Several factors affect competition of microbial communities such as pH, substrate preferences and affinity, and bacterial energy requirements (Russell and Baldwin, 1979). When

under extreme conditions, the bacteria most suited to the present environment will prevail until conditions change, such as an over accumulation of metabolites.

Bacteria have developed mechanisms to weaken their competitors to accomplish dominance in the normal environment. One such method is the excretion of antimicrobial substances with antagonistic effects of species in similar niches. These substances are referred to as bacteriocins (Russell and Baldwin, 1979). Notably, *Streptococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp. yield bacteriocins to inhibit the growth of one another and produce amines and proteinases that may elicit pathogenesis of host diseases discussed later in further detail (Foulquié Moreno et al., 2006; Harlow et al., 2015). Therefore, studies have been conducted to evaluate extreme conditions and host factors such as age, diet, feed management practices, body condition, and dietary state.

### **Microbial Related Gastrointestinal Disruption**

Diseases caused by disruption of colonized bacteria provoked further investigation into the ecosystem within the equine hindgut. Collectively, bacteria associated with the subsequent lameness observed in horses with laminitis has been characterized as Gram positive, bacteria with a cell wall made of layered peptidoglycan. A comprehensive list of causative agents leading to the pathogenesis of laminitis has not been completely elucidated, but microbial disruption in the hindgut from fermentation of carbohydrates provides a known method of induction (Bailey et al., 2002; Milinovich et al., 2006).

Laminitis has been described as ischemia of digital lamellae causing the failure of attachment between the distal phalanx and inner hoof wall. These events preclude the distal phalanx's progressive drive further into the hoof crushing surrounding circulation causing the

clinical symptoms seen in horses with acute laminitis (Pollitt and Daradka, 1998). A decrease in pH as a result of fermentation products may lead to increased intestinal permeability through loosely bound pre-keratinized epithelial cells allowing passage of bacteria or bacterial metabolites such as endotoxins, amines, and proteinases, to enter the blood stream (Harlow et al., 2014).

Excess quantities of dietary carbohydrates, especially exceeding 0.4 percent BW, are likely to overload the pre-ileal digestive and absorptive capacity, commonly demonstrated in an abrupt dietary transition. Within the vast ecosystem of the cecum, bacterial communities await the opportunity to transform the environment from symbiotic to highly competitive. Entrance of readily fermentable carbohydrates to the cecum provides a substrate that enables saccharolytic bacteria to flourish. As a consequence, high concentrations of lactate are accumulated, driving the environment to extreme conditions with a declining pH (5.9) (Goodson et al., 1988; Julliand et al., 2001; de Fombelle et al., 2003; Harlow et al., 2015; Harlow et al., 2016). In the absence of an adaption period, LUB are present in small numbers with a low capacity to ferment lactate, lagging in development in comparison to their starch-utilizing counter-parts. This results in the accumulation of lactic acid and concurrent pH drop (Harlow et al., 2015).

Although given a linear appearance, hindgut acidosis is the product of a cascade more closely resembling a positive feedback loop initiated by the *Streptococcus bovis/equinus* complex and extrapolated by the advancing decline of pH. In the quest to reveal the pathogenesis leading to laminitis, Milinovich et al. (2006) demonstrated a transition in the hindgut microflora from primarily G- to one dominated by G+ bacteria in horses with oligofructose induced laminitis within 18h. Lameness was observed in all horses on trial, however, 3/5 horses exhibited acute lameness 24h after administration of oligofructose as fecal pH reached the lowest point.

The remaining horses followed suit 6-12h later. Prior to the onset of laminitis, Milinovich et al., (2006) reported the abundance of bacteria in the *Streptococcus bovis/equinus* complex was the most prominent with the highest abundance of G+ cocci 16h post oligofructose administration. In support of the aforementioned study, Bailey et al. (2003) described an increased number of *Streptococcus* spp., *Lactobacillus* spp., high concentrations of lactic acid, and reduced pH when starch was added to an in vitro fecal suspension. However, the addition of a streptogramin antibiotic (virginiamycin) reduced the number of *Streptococcus* spp., effectively diminishing the negative outcome seen in the control.

A member of the Veillonella family, *Megasphaera elsdenii* has been implicated in pacifying the falling pH of rumen acidosis (Chen et al., 2019). The addition of this G- bacteria promotes the conversion of lactate to butyrate through the acrylate pathway in normal to sub-acute rumen acidosis states (pH  $\geq$  5.0). However, in acute rumen acidosis (pH < 5.0), *M. elsdenii* loses efficacy (Chen et al., 2019).

## **Diets Influence on Microbial Populations**

Diet, as well as meal size and frequency, play a crucial role in the structure of the ecosystem found in the hindgut (Venable et al., 2017). As a grazing species, horses have evolved a digestive tract equipped for a continuous influx of roughage. Nevertheless, when a forage diet no longer meets the animal's energy requirements, concentrate is commonly added as an energy dense supplement. Though management practices, including meal size and frequency, should be given special consideration. A decrease in meal frequency impacts the hindgut microflora reportedly causing fluctuations in *Streptococcus* and *Lactobacillus* spp. (Venable et al., 2017). Several studies (both in vivo and in vitro) have demonstrated changes in equine microflora in

response to diet composition. Collectively, as the proportion of concentrate to forage increases in the diet, the number of amylolytic bacteria (*Streptococcus* and *Lactobacillus* spp.) are rapidly increased (within 5h of ingestion); however, the extent is dependent upon the starch source (Goodson et al., 1988; Julliand et al., 2001; de Fombelle et al., 2003; Harlow et al., 2015; Harlow et al., 2016). When abruptly transitioned to 50:50 barley to timothy hay mix (Goodson et al., 1988; de Fombelle et al., 2003) reported a spike in total bacteria and amylolytic bacteria while LUB remained constant. This charade was succeeded by a peak in LUB 3-7 d post transition representing 69 percent of total bacteria followed by a reduction to 33 percent between the second and sixth week, a noted 10 percent higher than the initial abundance on the forage only diet. Incidentally, lactate concentrations increased significantly in the large intestine 5 and 29h after the dietary inclusion (de Fombelle et al., 2003). Contrarily, a depression in cellulolytic bacteria is observed as the ratio of starch is increased in the diets. Horses fed a diet high in oat or corn starch exhibited 10 and 1000-fold fewer cellulolytic bacteria from the control, respectively (Harlow et al., 2016). However, Kristoffersen et al. (2016) describes a peak in the *Fibrobacter succinogenes* population 4h after the incorporation of barley to an equine diet, which corresponds to substrates entrance in the cecum. This anomaly was speculated to be the result of the degradation of glycogen stored within *F. succinogenes*.

As previously mentioned, the severity of the response due to starch overload is dependent on starch source as well as the amount digested enzymatically. *Streptococcus* spp. hastily catabolize sugars producing copious amounts of lactic acid. Initially, the increase in a readily fermentable substrate allows for rapid growth of *Streptococcus*. However, *Streptococcus bovis* leads to its own demise through the production of the metabolite lactate causing pH to drop below 6.0, at which *Streptococcus* spp. cease to proliferate (Harlow et al., 2016). As the

proportion of barley increased in a study performed by Julliand et al. (2001), the concentration of lactate increased 10-fold. Goodson et al. (1988) reported that pH reached the lowest point (5.9) 7h after concentrate was consumed. Feeding corn starch incites the most demarcated responses in hindgut microflora with the lowest pH and highest measured lactate concentrations when compared to oats and wheat (Harlow et al., 2016).

The rapid decline in pH provides an opportunity for highly adaptive *Lactobacillus* spp. to burgeon, contributing to the further accumulation of lactic acid and cellular death of bacteria intolerant to low pH. *Lactobacillus* is a diverse genus of bacillus found throughout the equine GIT. It was once postulated that *Helicobacter pylori* was responsible for causing gastric ulcers in horses. Since, *H. pylori* has been vindicated as evidence has been found pointing to *Lactobacillus* spp. Throughout the glandular and non-glandular regions of the stomach and especially concentrated within ulcerated mucosa, *Lactobacilli* are found producing volatile fatty acids and lactate (Perkins et al., 2012). Unfortunately, absorption of VFAs are negligible in the stomach.

Interestingly, it has been demonstrated that the addition of oat versus corn starch promoted different predominant amylolytic bacteria (*Streptococcus bovis* and *Enterococcus faecalis*, respectively) as well as differences in the chronological trends corresponding to the growth, stationary, and death phases of bacteria (Harlow et al., 2015). After a 24h incubation period of fermentation, 100-fold more *Lactobacillus* spp. were found in oat starch fermentations when compared to corn starch. Additionally, G<sup>+</sup> cocci exhibited a 100-fold increase in both oats and corn starch fermentation; however, nearing the stationary phase, these numbers declined in only the oat incubations. Finally, there were 1000-fold more lactate utilizing bacteria in the oats compared to corn incubations after 24h. After 2h incubation, LUB displayed a 10-fold increase

in the oat incubation and 5-fold decrease in corn incubations. At 4h, both starch source incubations had decreased 50-100-fold then corn LUB numbers remained constant while oats began to rise. In an in vivo trial, corn and oat diets were formulated based on starch source at 2g/kg BW and fed to horses to determine starch sources' effect on the hindgut microflora employing 16s RNA sequencing. Here, it was established that corn elicited the most amylolytic bacteria (10,000-fold greater than oats), expressing more G+ cocci and fewer *Lactobacillus* spp. and LUB than oats. The lack of LUB found in corn in relation to oats explains the significant accumulation of lactate and pH decline measured in this trial, subsequently followed by a declining population of cellulolytic bacteria (Harlow et al., 2016).

Activity and composition of the microbial community in relation to segments of the GIT is a result of substrate supply and GIT anatomy (Dougal et al., 2012). Once masticated in the mouth, fresh digesta enters the acidic environment (pH= 2.0) of the stomach, presumably extinguishing bacteria that are seen in the more distal gut; yet, *Lactobacillus* spp. and *Streptococcus* spp. have been recovered from its interior. However, changes in the gastric microbiome due to diet have not been well characterized or recognized as a principal factor of digestion (Perkins et al., 2012). Using next generation sequencing techniques, Ericsson et al., (2016) determined the foregut is a region of high variability within and between horses, limiting the ability to cluster bacteria into functional groups. Ericsson et al., (2016) further revealed a blatant contrast between the microbiome in the ileum and cecum, suggesting the differential anatomy demarcates the transition to digestion ruled by fermentation. Here, digesta rich in cellulose or resistant starch enters the cecum then colon. The presence of hindgut bacteria, specifically fibrolytic species, is crucial for these herbivores to survive (Shepherd et al., 2012). In proportion to their importance, the majority of dietary related changes to the microbial



populations are seen in the hindgut, mainly, the colon (Julliand et al., 2001; Dougal et al., 2012). Such diet related transitions have been reported to take as few as 4d (Fernandes et al., 2014); however, other studies indicate a one- week adaption period necessary for a complete transition of the microbiome (Earing et al., 2010). Previously, Dougal et al. (2014) discovered strong agreement in the microbiome within the cecum and colon between horses supporting the notion brought forth by Lowman et al., (1999), suggesting the similar composition of feces to the distal hindgut validates the use of a fecal inoculate for in vitro studies. Lowman and colleagues (1999) demonstrated that equine feces are usable hours after excretion and provide a viable and accurate microbial inoculate for laboratory purposes. Presently, the use of feces to inoculate in vitro digestion systems is widely accepted as a true representation of the microbial ecosystem through in vivo processes and to produce accurate results.

### **Estimating Digestibility**

Diet digestibility is a function of intrinsic properties and intramolecular composition of plant cell walls (Mertens, 1993). Ruminant producers have used relative feed value (RFV) to compare forages; however, RFV provides a poor estimation of animal performance as it uses an estimate of digestibility and filling capacity in the prediction (Hansen and Lawrence, 2017). A more appropriate estimation, relative forage quality (RFQ), has been constructed to predict performance, which uses total digestible nutrients and intake as a percent body weight. Relative forage quality has been shown to predict performance in ruminants; however, the application of RFQ is undetermined in horses and does not account for interactions between feedstuffs (Kienzle et al., 2002).

Rate of passage, defined as the flow of material within or through the entire digestive tract per unit of ingesta, has been demonstrated to affect digestion and digestive disorders (van Weyenberg et al., 2006; Huhtanen et al., 2009). Thus, digestibility is not only a factor of chemical structure but physical form, intake quantity, and interactions in mixed diets. Reports indicate that decreased particle size and increased dietary intake result in an increased rate of passage, ultimately reducing digestibility (van Weyenberg et al., 2006; Huhtanen et al., 2009; de Souza et al., 2018).

Several studies have attempted to reveal a predictive equation in which a set of nutrients would accurately outline the performance of equine and ruminants. Thus far, NDF content has been the most highly correlated component with dry matter digestibility (DMD) consistent across equine and ruminant species with crude protein (CP) to follow (Du et al., 2016; Hansen and Lawrence, 2017; de Souza et al., 2018). (Du et al., 2016), reported a strong negative correlation between NDF and ADF and NDF digestibility (ADFD and NDFD) while CP showed a positive correlation, suggesting these components may be used in the future to predict DMD, NDFD, and ADFD of forages. Supporting the previous statement, de Souza and associates expressed that the most accurate equation elucidated in the (2018) study was based on NDF in which dietary and intake variables were included. This equation, calculated to fit a ruminant model, accounted for grass and starch as a percent dry matter, and linear and quadratic effects of dry matter intake as a function of body weight. The necessity to include fiber and starch provides evidence for a possible interaction between feedstuff. It was also reported in this study that for every 1 percent increase in dietary fiber, NDFD was decreased by 0.59 percent suggesting a depression in fiber digestion with the addition of starch. Conversely, there have been reports of decreased digestibility of organic matter when starch was replaced with fiber (Swyers et al., 2008;

Nousiainen et al., 2009). Further, when horses were fed a diet composed of low-quality straw hay (CP=2.1) the addition of concentrate increased the digestibility of the forage component (Kienzle et al., 2002). It is possible that the extremely low dietary CP lacked the nitrogen necessary to maintain a healthy hindgut microflora; therefore, the concentrate restored the microbial population and subsequent fermentative capacity.

A study by Hansen and Lawrence (2017) compiled the data from previous studies and created a regression equation using NDF, ADF, CP, hemicellulose, and lignin to determine the strongest correlation between variables and dry matter digestibility. Twenty-six in vivo digestibility studies on mature horses or ponies (>2 years of age) fed an all forage diet, reporting NDF, ADF, CP, and DMD were used, resulting in data for 70 forage diets. A variety of legume hay, warm and cool season grasses were distributed through the reported studies. Other variables, such as Hemicellulose (HEM) and percent ADF in the NDF fraction (PER) were calculated as  $HEM = NDF - ADF$  and  $PER = ADF / NDF \times 100$ . Relationships between forage composition and DMD were determined with both simple linear and multiple regressions using the following variables: NDF, ADF, CP, HEM and/or PER. Many significant equations ( $p < .0001$ ) arose, however, the most promising,  $DMD = 65.81 + .7207 \times CP - 0.3514 \times NDF$  (Adj.  $R^2 = .6583$ ), suggests the variables CP and NDF are the best fit to determine DMD in the data presented (Hansen and Lawrence, 2017). Even so, the outlined study evaluated equine on forage only diets and did not compare diets which included grain.

Therefore, a study was designed with the objectives to evaluate the forage parameters and the effect of fecal donor diet on the digestibility of NDF, ADF, and DM. In addition to expanding the database of digestibility trials, the purpose of the study was to determine the difference in digestibility of neutral detergent fiber, acid detergent fiber, and dry matter when

horses are fed a forage only diet and a high grain: forage diet, and determine if NDF, ADF, and CP could be used to predict fiber digestibility.

## MATERIALS & METHODS

### Experimental Design

Six mature Quarter Horses ( $n=6$ ; 3 mares and 3 geldings) were selected from Missouri State University's herd located at Pinegar Arena 2401 S Kansas Expressway Springfield, Missouri. Horses weighed  $522\pm45\text{kg}$  and ranging from  $14\pm9$  years of age. All procedures involving the care, management, and use of equine in this study was approved by the Institutional Animal Care and Use Committee of Missouri State University (IACUC#2018-12). Horses were sorted into one of two groups, G1 or G2. Horses elected to receive the grain diet for the first period were geldings previously on a mixed concentrate diet and housed in 3 x 3m stalls. Three mares in the opposing group were previously turned out on a fescue mixed grass pasture. The number of horses used in the trial were selected based upon previous research in which the same or fewer subjects were used (Kienzle et al., 2002  $n=4$ ; Milinovich et al., 2006  $n=5$ ; Harlow et al., 2015  $n=6$ ). During the trial, horses were kept in 3 x 3.6m stalls with limestone-based footing. Horses had ad libitum water and access to a mineral block. Feedings took place at 0700 and 1900h with refusals measured 12h post feeding. Both groups were exercised lightly, as outlined by the National Research Council (2007), throughout the duration of the study.

**Treatments.** Treatments included a mixed grain + forage ration (GF) and a forage only (F) diet. Corn chops were chosen as the cereal grain in the present study for its strong impact on equine hindgut microbiota (Harlow et al., 2015; Harlow et al., 2016). Corn chops (Mainstreet Feeds Inc. Springfield, Missouri), commercially available alfalfa cubes ("Top Of The Rockies", Manzanola Feeds, Manzanola, Colorado) and a fescue mixed grass hay, harvested in Springfield, Missouri, were fed at a 1:1 (forage: concentrate) ratio for the GF diet while horses on diet F

consumed alfalfa cubes and fescue mixed grass hay. Both diets were fed at 2 percent body weight (BW) formulated to meet or exceed the horse's energy requirements for moderate exercise. Table 1 lists the nutrient analysis of dietary components and respective ratios in diets on a dry matter basis. Dietary crude protein in the rations was within 0.1 percent dry matter, comparatively. In the first 22-day time-period, G1, initiated the study on diet F while G2 started with a gradual introduction to the GF diet to avoid digestive upset. Horses in G2 were acclimated to the diet by increasing the starch source by 25 percent increments every 72h as outlined by (Harlow et al., 2016). Such that, by the second feeding on day 10, G2 consumed 100 percent of the treatment diet. On day 22, the groups switch treatments, and G1 started the acclimation process (Figure 2).

**Forage Samples.** Prior to the first sampling period, hay samples were taken from three hays (legume/mixed grass hay, fescue mixed grass hay 1, and fescue mixed grass hay 2) harvested within an 80 km radius of Darr Agriculture Center, Springfield, Missouri. As there was little concern for the true representative values of the harvested cuttings, only hay used in the treatment diet of the study was sampled by coring 20 individual bales. However, continuity of hay quality between sampling periods was imperative. Therefore, remaining samples were procured from a flake (~2kg) of a randomly selected bale and 15 cubes from three 22kg of the same commercially available bags of alfalfa cubes. Forages were processed individually, each sample representing hay of similar quality. Forage samples were sent to Custom Labs in Golden City, Missouri, for ADF, NDF, and CP analysis. Results of their forage analysis are listed in Table 2.

In the laboratory, dry matter was calculated based on weights recorded before and after drying a subsample of forages in a 50°C oven for 48h using the following equation: (dry sample weight (g) / wet weight (g)) x 100 = percent dry matter. Prior to grinding, individual forage samples were homogenized with an Oster® food processor until 150 g of the sample's particles were smaller than 5 mm in length. The Cyclone Mill-Direct Drive™ (UDY Corporation, Ft. Collins, Colorado) processed hay samples further to pass through a 1 mm screen. After processing was complete, hay samples were stored in sealable freezer bags.

In preparation for each sample period, F57 filter bags (Ankom Technology, Macedon, New York) were soaked in acetone for five minutes and allowed to air dry to dissolve antimicrobial coating as per manufacturer instructions. Subsequently, filter bags were labeled and weighed. Labels included identification of fecal donor used in the individually prepared inoculate, sample period, hay sample code, and an indication of the duplicate. A blank bag was also included in each digestion jar to account for corrections during the calculations. Next, eight filter bags were loaded with 0.45-0.5g of processed forage samples per fecal inoculate. All four forage samples were present, in duplicates, in digestion jars each containing fecal inoculates prepared from one fecal donor. Filled filter bags were closed with the 120V Impulse Heat Sealer less than 4mm from the top to allow even dispersal and exposure throughout the bag.

Prior to fecal sample collection, the buffer solution responsible for mimicking the physiological environment of the equine cecum was prepared and allowed to equilibrate for a minimum of 30 min before incubation. The buffer solution was made of the following reagents following the Daisy II manufacturer instructions (Ankom Technology, Mecedon, NY): in a 5000mL beaker, solution A was started by bringing 1330mL of distilled water to 50°C and adding  $\text{KH}_2\text{PO}_4$  at 10.0g/L;  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  at 0.5g/L; NaCl at 0.5g/L;  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$  at 0.1g/L;

and urea at 0.5g/L. Once solution A was homogenized, 266mL of solution B, containing Na<sub>2</sub>CO<sub>3</sub> at 15.0g/L and Na<sub>2</sub>S·7 H<sub>2</sub>O at 1.0g/L, was added to the 5000mL beaker and titrated to a pH of 6.8 at 39°C. Reagents for the mixture were purchased from Sigma-Aldrich, St. Louis, MO. The solution was poured into a digestion jar and allowed to equilibrate while fecal samples were collected.

**Fecal Sample Collection.** Fecal samples were collected via rectal palpation on days 0, 11, 22, 33, and 44 (Figure 3). Day 0 acted as a baseline for subsequent measurements and day 22 served the dual purpose of the last data entry for the first diet and baseline for the second. However, due to the unexpected limited range of analyzed forage samples for the in vitro trial, alfalfa cubes were not added to the suite of forage samples until day 11, thus, a baseline could not be established as the statistical analysis could not estimate missing samples. Samples were collected at 1300h, between meal-times, and immediately deposited in an air-tight plastic bag, then stored in a pre-warmed Igloo™ Cooler at 39° C until processed in the lab. Once in the lab, each fecal sample was prepared independently. A blank bag and duplicate filter bags for each forage sample were present in each digestion jar per incubation (n = 9), with the exception of day 0, in which the alfalfa cubes were absent (n=7). Samples were analyzed for DM, NDF, and ADF digestibility using methods developed by Van Soest (1970) and adapted by Ankom Technology (1998). First, 40g of the fecal sample was added to an Oster® blender containing 400mL of distilled water at 39°C. The blender was continuously purged with CO<sub>2</sub> while running on the liquify setting for 25 seconds. Next, the suspended sample was strained through two layers of cheesecloth to rid the inoculate of excess particles. Four-hundred milliliters of inoculate was poured into the digestion jars containing 1,600mL of buffer solution, loaded filter bags were



added, and the system was purged again with CO<sub>2</sub> for 30s before the jar was sealed and allowed to incubate.

In vitro digestibility trials were conducted with the assistance of the Ankom Daisy II Incubator (D200, Ankom Technology, Mecedon, NY). As the fermentation jars were prepared for incubation, the Daisy II Incubator preheated to 39°C. After the 48h incubation, jars were removed, and the filter bags were rinsed with cold tap water to stop microbial activity as described by (Lattimer et al., 2007). Manual agitation and manipulation of the filter bags were performed during the rinsing process to ensure all digested particles and by-products were removed from the bag prior to drying and re-weighing. Rinsed filter bags were dried in a 50±2°C oven for 48h before being weighed to determine DMD. Bag correction factor is calculated by dividing the final oven dried bag weight by the initial dry blank bag weight. In vitro dry matter digestibility was calculated using the following equation: 
$$[(100 - (\text{final dried weight} - (\text{initial empty bag weight} \times \text{correction factor}))) \div (\text{initial sample weight} \times \text{dry matter})] \times 100 = \text{percent in vitro DMD}.$$

For a more thorough understanding of the fraction of dry matter digested through fermentation, the Ankom Fiber Analyzer (ANKOM<sup>2000</sup>, Ankom Technology, Mecedon, NY) was used to assess digested NDF and ADF in the sample. As ADFD removes hemicellulose, NDFD analysis was completed first. The dried filter bags were placed into the cylinder of the fiber analyzer, water at 70°C flushed into the vault, and agitation began with the addition of NDF solution. Neutral detergent solution concentrate (Ankom FND20C) diluted by 20L of distilled water and the accompanied triethylene glycol was mixed in 5L aliquots, then combined in an Ankom Cubetainer assembled to the fiber analyzer for administration. Furthermore, filter bags loaded with the initial finely milled forage samples and a blank bag, prepared as previously

described, were added to the chamber of the fiber analyzer. Manually, 20g of sodium sulfite (Ankom FSS) and 4mL of  $\alpha$ -amylase (Ankom FAA) were added to the agitating chamber prior to sealing the system. Eight milliliters of  $\alpha$ -amylase diluted by 250mL of distilled water was poured into the secondary portal for subsequent rinses. The addition of  $\alpha$ -amylase prevents starch contamination in the event starch remains after in vitro digestion, which would lead to the misrepresentation of percent NDF (National Research Council, 2007). Upon cycle completion, the filter bags were removed, excess water was pressed out, and they were inserted into an acetone filled 250mL beaker to soak for 3-5 minutes. After the acetone bath, the samples were laid to air dry on a tray until the acetone evaporated then dried in a  $100\pm 2^{\circ}\text{C}$  oven for 2-4h. Dry filter bags were removed from the oven and stored in Ankom MoistureStop Weigh Pouches (Ankom X45, Ankom Technology) until internal temperatures reach approximately room temperature ( $25\text{-}26^{\circ}\text{C}$ ). Bags were weighed again to analyze percent digested NDF.

Acid detergent-liquid concentrate (Ankom FAD20) diluted by 20 L distilled water was pre-made and poured into an Ankom Cubetainer, then attached to the ADF Solution Portal of the ANKOM<sup>2000</sup>. In close resemblance to the process preformed for NDF analysis, the dried filter bags were loaded into the chamber of the fiber analyzer and the cycle begins; however, no extraneous chemicals or enzymes were added. After the cycle, the bags underwent the same treatment as previously described in NDF analysis. Final weights were recorded, and percent digested ADF was determined.

Table 1. Experimental diet composition and nutrient analysis of neutral detergent fiber, acid detergent fiber and crude protein (DM basis) of feedstuff in treatment diets and respective ratios found in the forage and grain diets.

	Treatment Diets			
	%NDF DM	% ADF DM	% CP DM	Ratio in ration
<b>Grain Diet (GF):</b>				
Cracked Corn	9.5	3.4	9.4	0.5
Fescue Mixed Grass Hay 2	72.7	63.2	7.7	0.39
Alfalfa Cubes	53	44	16.4	0.11
Diet Total:	38.9	31.2	9.5	1
<b>Forage Diet (F):</b>				
Fescue Mixed Grass Hay 2	72.7	63.2	7.7	0.81
Alfalfa Cubes	53	44	16.4	0.19
Diet Total:	68.9	59.6	9.4	1

Forage parameters: crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using wet chemistry and NIRs spectrometry. All diets fed at 2 percent BW DM basis.

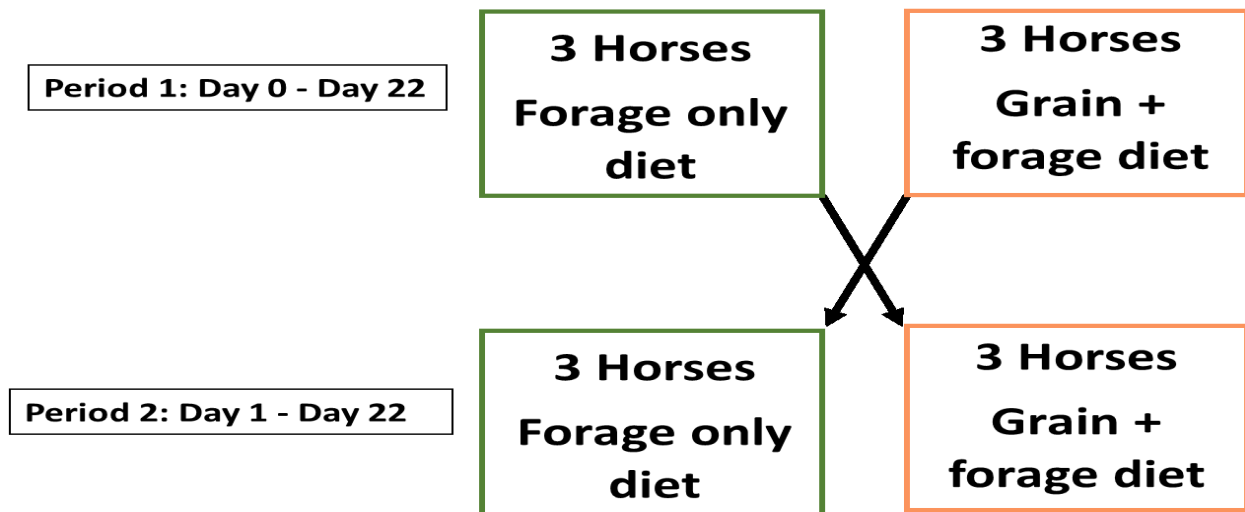


Figure 2. Depiction of treatment protocol: six horses divided into two groups and assigned the grain or forage diet for the first 22-day period and subsequent dietary transition to the opposing diet for the second 22-day period.

Table 2. Neutral detergent fiber, acid detergent fiber, and crude protein analysis of forage samples evaluated in the in vitro digestibility trial within the Daisy II Incubator using fecal inoculates collected from experimental subjects on treatment diets.

	Forages Digested with Daisy <sup>II</sup> Incubator in vitro			
	Alfalfa Cubes*	Legume + Mixed Grass Hay	Fescue Mixed Grass Hay 1	Fescue Mixed Grass Hay 2*
%NDF DM	53	67	71.1	72.7
%ADF DM	44	59	61.8	63.2
%CP DM	16.4	10.3	8.6	7.7

\* Forages used in treatment diets. Forage parameters: crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using wet chemistry and NIRs spectrometry. All diets fed at 2 percent BW DM basis.

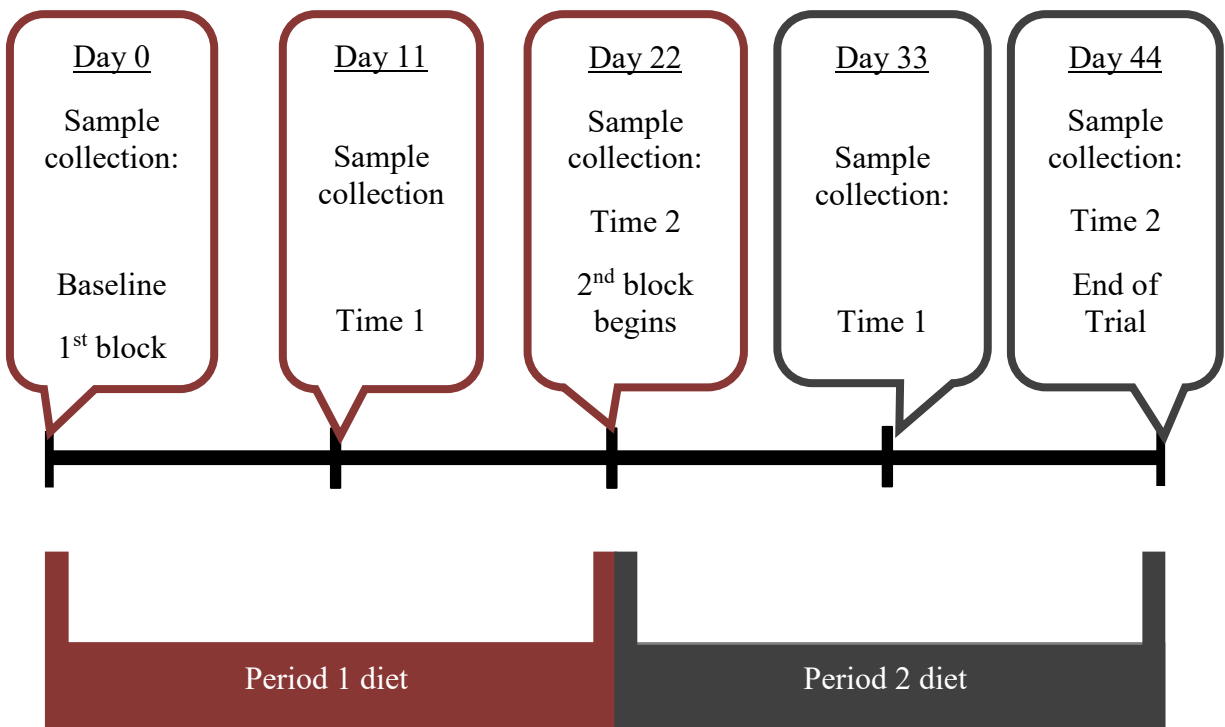


Figure 3. Illustration of fecal sample collection schedule. The baseline, Time 1 and Time 2 are divided by period.

## Statistical Analysis

Data collected from fiber digestion within the digestion jars of the Daisy<sup>II</sup> Incubator were analyzed as individual products of a fecal inoculate pulled from one subject. Duplicates within jars were averaged for a more accurate assessment. Data from day 0 were excluded from the analysis as alfalfa cubes were not added to the suit of forages analyzed until day 11; consequently, a baseline could not be established. When graphed, a time x diet within forage interaction was not apparent (Figure 4); however, insufficient degrees of freedom prohibited the evaluation of the statistical significance. Nevertheless, time 1 was also excluded from further analysis. Time 2 data were analyzed using SAS (version 9.2, SAS Institute, Inc. Cary, NY) PROC MIXED procedure testing the effect of covariance parameters (CP, NDF, and ADF) on the digestibility of DM, NDF, and ADF. The covariate test allows use of numerical covariates in the analysis negating the subjectivity associated with categorizing forages into high, medium, and low qualities. Class statement included: horse, period, diet, forage quality, and sequence. In the model, horse within period by sequence was included as a random effect to account for variation due to experimental error, individuality, and other environmental factors that may have contributed to differences measured during sampling times. Parameters measured are analyzed based upon the differences in slope across forages and significance was set at  $\alpha = 0.05$  with trends at ( $p \leq 0.1$ ).

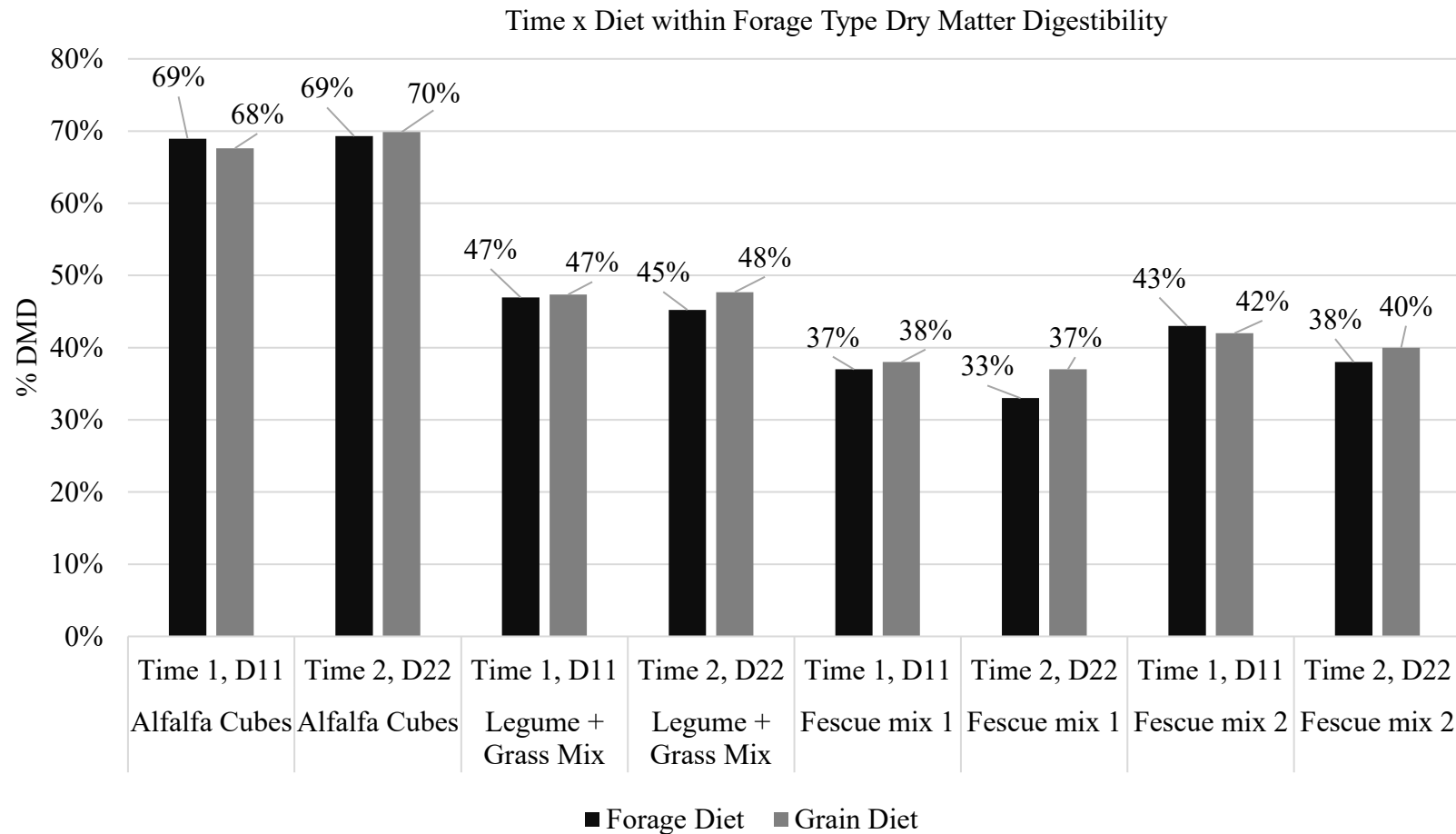


Figure 4. Bar graph of dry matter digestibility illustrating the absence of a time by diet within forage interaction. Black bars represent the forage dry matter digestibility from fecal inoculates collected from horses on the forage diet while the gray bars were collected from horses on the grain diet.

## RESULTS

### Individuality

Horse within period x sequence was included as a random effect in the analysis as the individuality between subjects significantly affected the digestibility in all DM and NDF analyses ( $p \leq 0.05$ ) and tended to affect digestibility of ADF ( $p \leq 0.1$ ). Tables 3,4, and 5 include the estimated percent variability DMD, NDFD, and ADFD, respectively, accounted for by the random horse effect when CP, NDF, and ADF were used as covariates. levels at all parameters (Figure 5). An example of the variation of DMD of a Fescue-mixed grass hay between individuals is represented in Figure 5.

Table 3. Mean percent variation due to horse in dry matter digestibility analysis using forage crude protein, neutral detergent fiber, or acid detergent fiber content as covariates.

	Horse within Sequence x Period	
	%Variation	P-Value
DMD		
CP	7.7±3.8	0.02*
NDF	7.6±3.9	0.02*
ADF	6.8±3.9	0.04*

\* Indicates significant P-value ( $P < 0.05$ ). Estimated percent variation± SEM due to individual horse differences, experimental error, or environmental factor that may have skewed data.

Table 4. Mean percent variation due to horse in neutral detergent fiber digestibility analysis using forage crude protein, neutral detergent fiber, or acid detergent fiber content as covariates.

	Horse within Sequence x Period	
	%Variation	P-Value
NDFD		
CP	10.6±5.7	0.03*
NDF	10.4±5.7	0.03*
ADF	9.4±5.7	0.05*

\* Indicates significant P-value ( $P < 0.05$ ). Estimated percent variation± SEM due to individual horse differences, experimental error, or environmental factor that may skew data.

Table 5. Mean percent variation due to horse in acid detergent fiber digestibility analysis using forage crude protein, neutral detergent fiber, or acid detergent fiber content as covariates.

Horse within Sequence x Period		
	%Variation	P-Value
ADFD		
CP	7.9±4.8	0.051
NDF	7.8±4.8	0.054
ADF	6.8±4.9	0.08

\* Indicates significant P-value ( $P < 0.05$ ). Estimated percent variation ± SEM due to individual horse differences, experimental error, or environmental factor that may skew data.



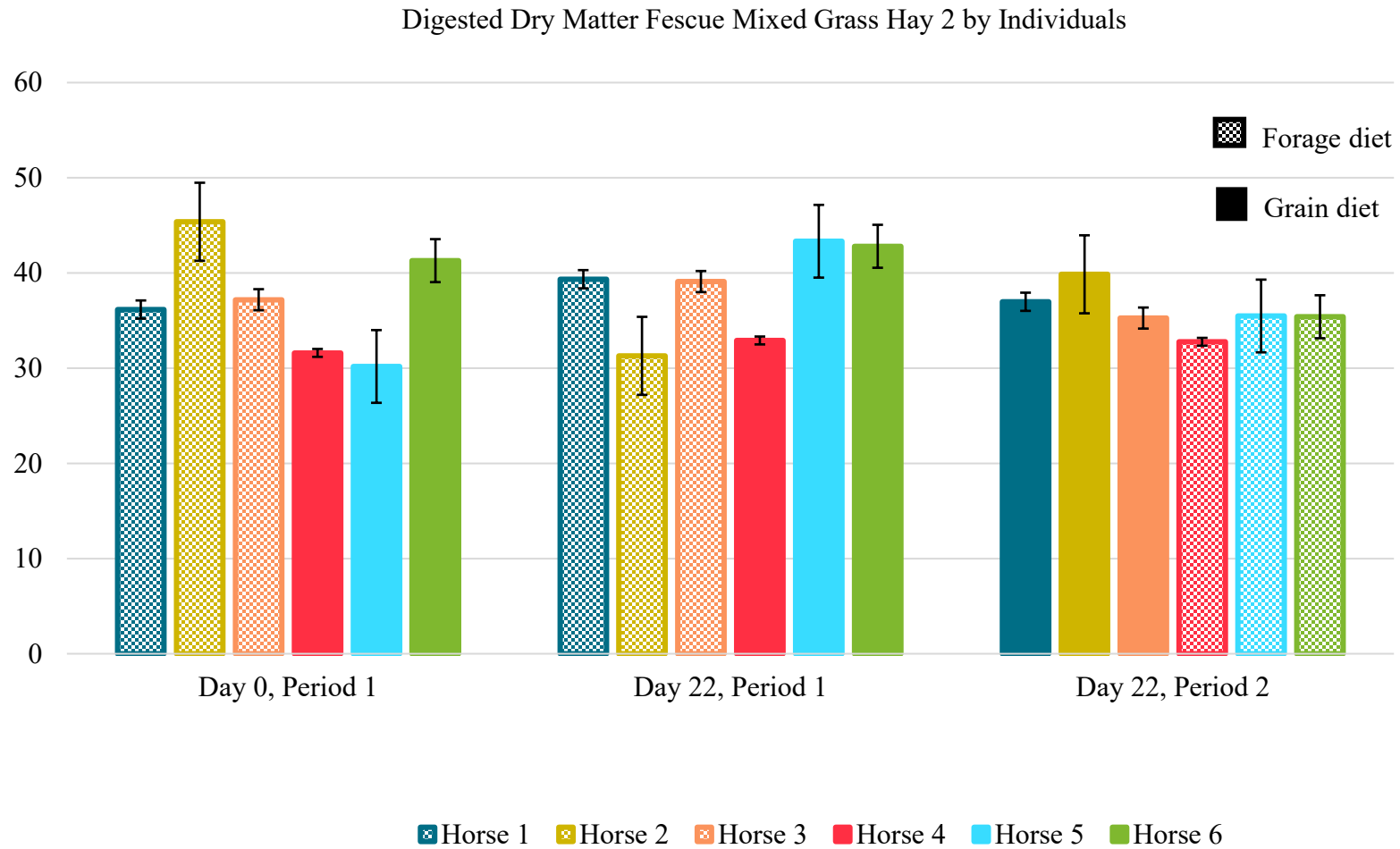


Figure 5. Individually digested dry matter (Mean  $\pm$  SEM) of fescue mixed grass hay number 2 on Day 0 and Day 22 in both periods. Individual horses are represented on the x-axis. Textured bars are horses on forage diet and solid bars are consuming grain.

## Digestibility Trials

**Acid Detergent Fiber Digestibility.** Means of forage samples, digested in vitro with a fecal inoculate then with acid detergent, and measured nutrient content within forages were evaluated per diet with covariate analysis. Slope between diets within measured forage parameters, including crude protein, neutral detergent fiber, and acid detergent fiber were compared and data listed in Table 6. In a linear regression, there was no interaction between diet x CP ( $P=0.14$ ). The same was true for NDF x diet and ADF x diet interactions ( $P=0.159$  and  $P=0.216$ , respectively). Graphed linear regressions with respective diet equations can be seen in Figures 6, 7, and 8.

**Neutral Detergent Fiber Digestibility.** Means of forage samples, digested in vitro with a fecal inoculate then neutral detergent, and measured CP, NDF, and ADF content within forages were evaluated per diet with covariate analysis. Slope between diets digestibility by measured forage parameters, including crude protein, neutral detergent fiber, and acid detergent fiber were compared and data listed in Table 7. A difference in diet x crude protein linear regression was apparent at ( $P=0.03$ ). Further investigation revealed a trend for 3.9 percent higher digestibility of forages with eight percent CP concentration when horses are fed a grain diet rather than forage only ( $P=0.08$ ). However, higher crude protein concentrations did not affect neutral detergent fiber digestibility. Slope of diet x percent NDF interaction was also significant at ( $P=0.04$ ). On diet G, horses tended to digest the neutral detergent fiber of forage with 72 percent NDF, 3.8 percent higher than horses on diet F ( $P=0.08$ ). Yet, NDFD of forages composed of 63 percent or less NDF were not different (Table 8). Composition of ADF showed a trend in NDFD ( $P=0.088$ ); however, as the interactions failed to meet the significance level, least square means were not included in the report. Graphed linear regressions with respective crude protein, neutral detergent fiber, and acid detergent fiber equations can be seen in Figures 9, 10, and 11.

**Dry Matter Digestibility.** Means of forage samples, digested in vitro with fecal inoculates, and previously assessed forage parameters were analyzed by diet with an analysis of covariance in PROC Mixed. Resulting linear regression evaluated slope of the digestibility by diets across forage parameters to determine statistical differences. Albeit statistically insignificant, a trend in the crude protein x diet interaction was measured ( $P=0.059$ ). There was a tendency for an increase of 3.2 percent digested dry matter of forages with  $CP \leq 8$  percent when horses were fed a grain diet ( $P=0.08$ ). Effects of neutral detergent fiber x diet interactions were also on the precipice of significance ( $P=0.06$ ). Here, another trend was recognized when forage  $NDF \geq 72$  percent. Table 9 provides the mean DMD values of the diet by CP, NDF, and ADF interaction to construct the regressed equations. When fed a grain diet, horses digested 3.1 percent more dry matter than horses fed a forage only diet ( $P=0.08$ ) (Table 10). Slope of acid detergent fiber x diet interactions did not differ ( $P=0.16$ ). Linear regressions with individually plotted DMD are represented in Figures 12, 13, and 14.

Table 6. Diet of fecal inoculate donor by crude protein, neutral detergent fiber, and acid detergent fiber regressions of acid detergent fiber digestibility analysis.

	Grain Diet		Forage Diet	
	Intercept	Slope	Intercept	Slope
CP	- 1.1 $\pm$ 2.5	2.9 $\pm$ 0.20	- 8.1 $\pm$ 2.5	3.3 $\pm$ 0.20
NDF	113.6 $\pm$ 6.2	-1.3 $\pm$ 0.09	123.6 $\pm$ 6.2	-1.5 $\pm$ 0.09
ADF	99.3 $\pm$ 6.1	-1.2 $\pm$ 0.10	107.5 $\pm$ 6.1	-1.4 $\pm$ 0.10

<sup>a,b</sup>Row means ( $\pm$ SEM) with different superscripts indicate significant differences in the slope ( $p < 0.05$ ). Forage parameters crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using wet chemistry and NIRs spectrometry. Digestibility parameters: acid detergent fiber digestibility (ADFD) was analyzed using the and Daisy<sup>II</sup> Incubator and Ankom<sup>2000</sup> Fiber Analyzer.

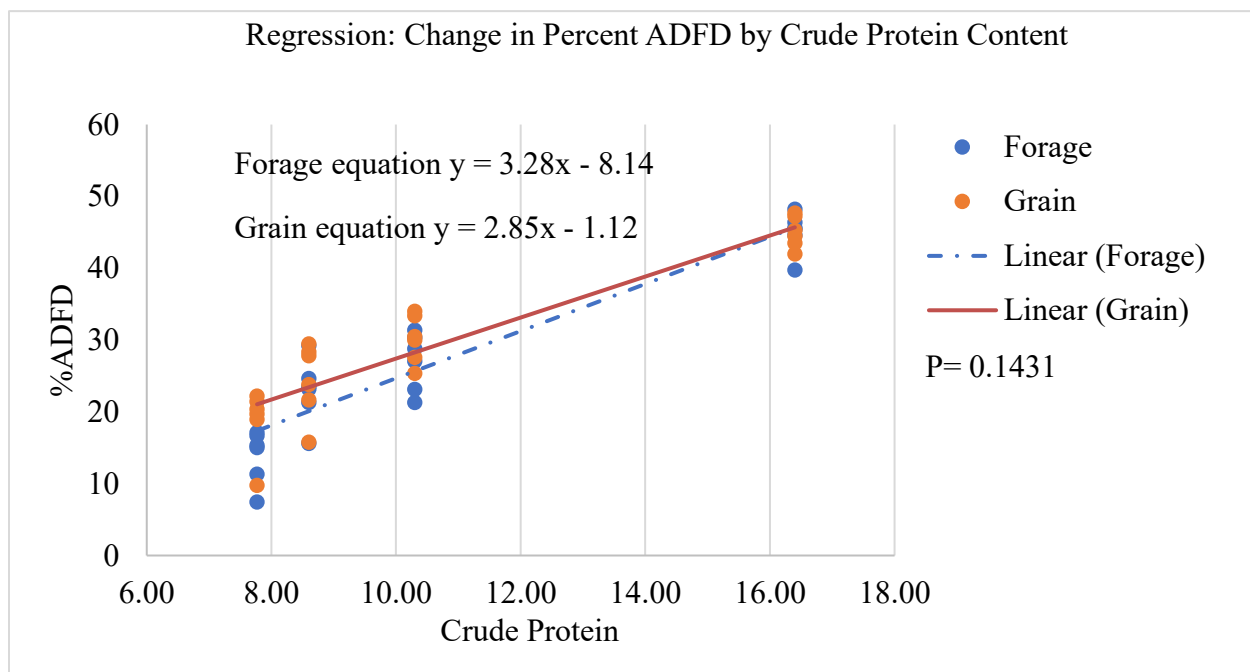


Figure 6. Diet of fecal inoculate donor by crude protein interaction in acid detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

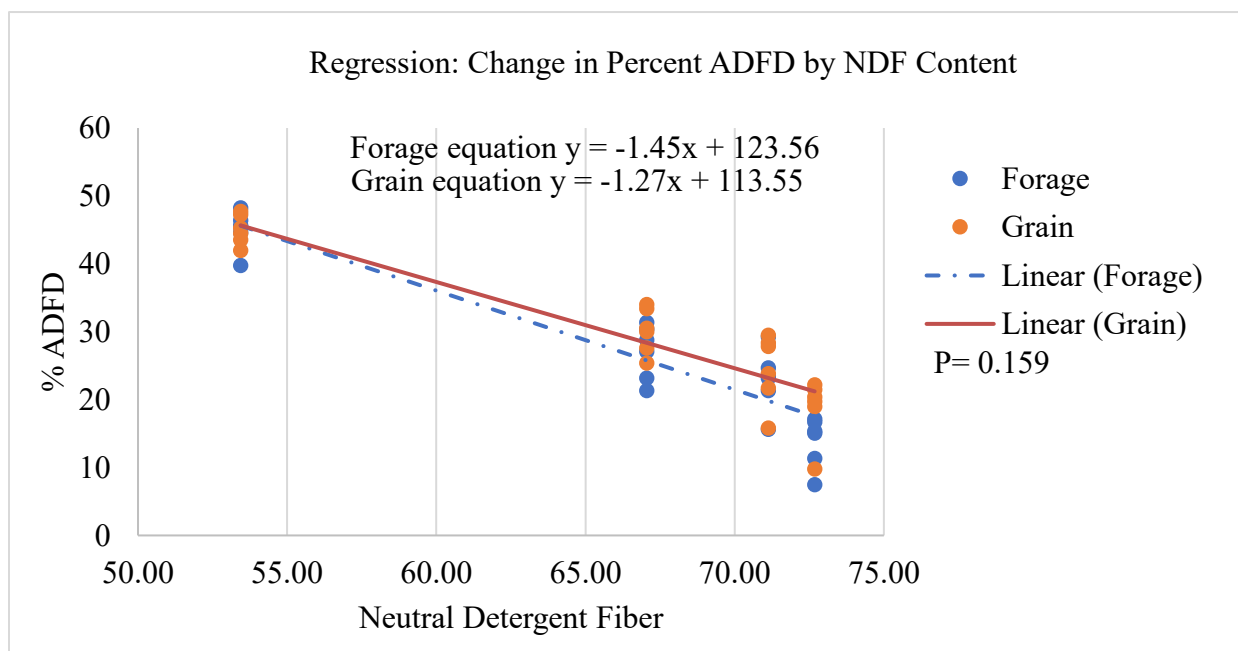


Figure 7. Diet of fecal inoculate donor by neutral detergent fiber interaction in acid detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

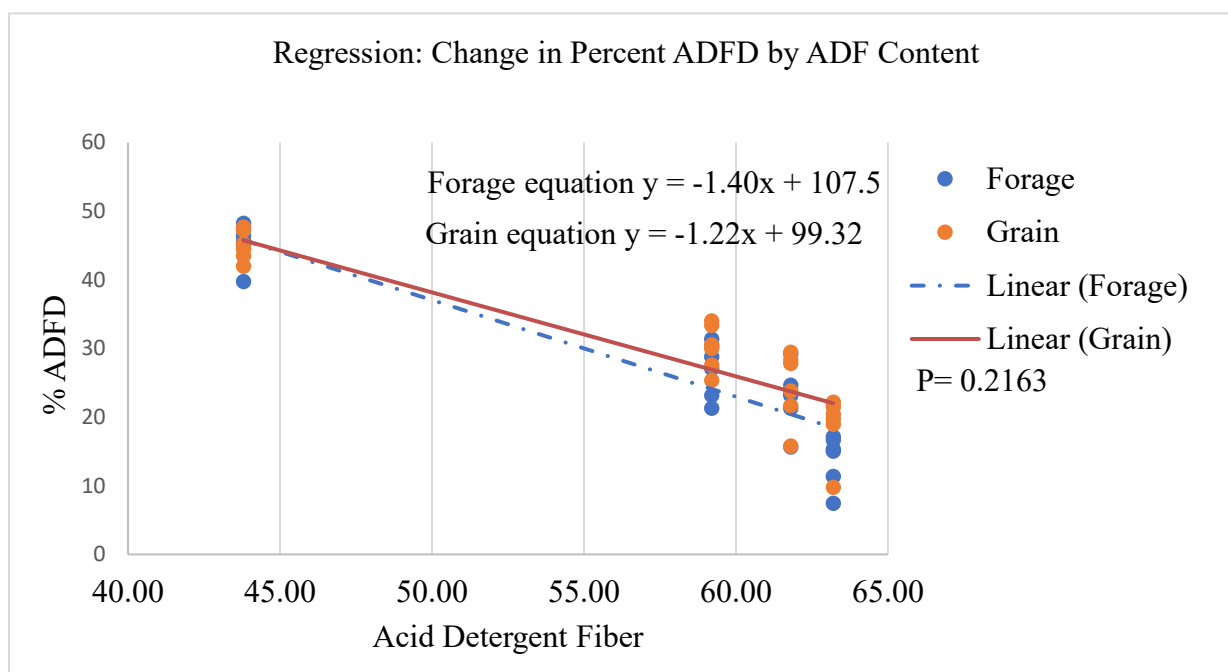


Figure 8. Diet of fecal inoculate donor by acid detergent fiber interaction in acid detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

Table 7. Diet of fecal inoculate donor by crude protein, neutral detergent fiber, and acid detergent fiber regressions of neutral detergent fiber digestibility analysis.

	Grain Diet		Forage Diet	
	Intercept	Slope	Intercept	Slope
CP	- 4.0±2.4	3.4±0.17 <sup>a</sup>	12.4±2.4	3.9±0.17 <sup>b</sup>
NDF	132.3±5.6	-1.5±0.08 <sup>a</sup>	146.0±5.6	-1.8±0.08 <sup>b</sup>
ADF	115.9±5.6	-1.5±0.09 <sup>a</sup>	127.0±5.6	-1.7±0.09 <sup>a</sup>

<sup>a,b</sup> Row means (±SEM) with different superscripts indicate significant differences in the slope ( $p < 0.05$ ). Forage parameters crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using wet chemistry and NIRs spectrometry. Digestibility parameters: neutral detergent fiber digestibility (NDFD) was analyzed using the Daisy<sup>II</sup> Incubator and Ankom<sup>2000</sup> Fiber Analyzer.

Table 8. Effect fecal donor diet by percent crude protein and fecal donor diet by percent neutral detergent fiber in dietary forages on neutral detergent fiber digestibility

	Grain Diet	Forage Diet	P-value
%CP			0.03*
16	50.2±1.7	50.7±1.7	0.84
12	36.7±1.5	34.9±1.5	0.42
8	23.1±1.5	19.2±1.5	0.08
%NDF			0.04*
72	23.5±1.5	19.7±1.5	0.08
63	37.1±1.5	35.5±1.5	0.43
54	50.7±1.8	51.3±1.8	0.82

<sup>a,b</sup>Row means (±SEM) with different superscripts indicate significant differences in the slope ( $p < 0.05$ ). P-values with an asterisk indicate significance ( $P < 0.05$ ). Forage parameters crude protein (CP) and neutral detergent fiber (NDF) were measured using wet chemistry and NIRs spectrometry. Neutral detergent fiber digestibility (%NDFD) was analyzed using the Ankom Daisy<sup>II</sup> Incubator and Ankom<sup>2000</sup> Fiber Analyzer.

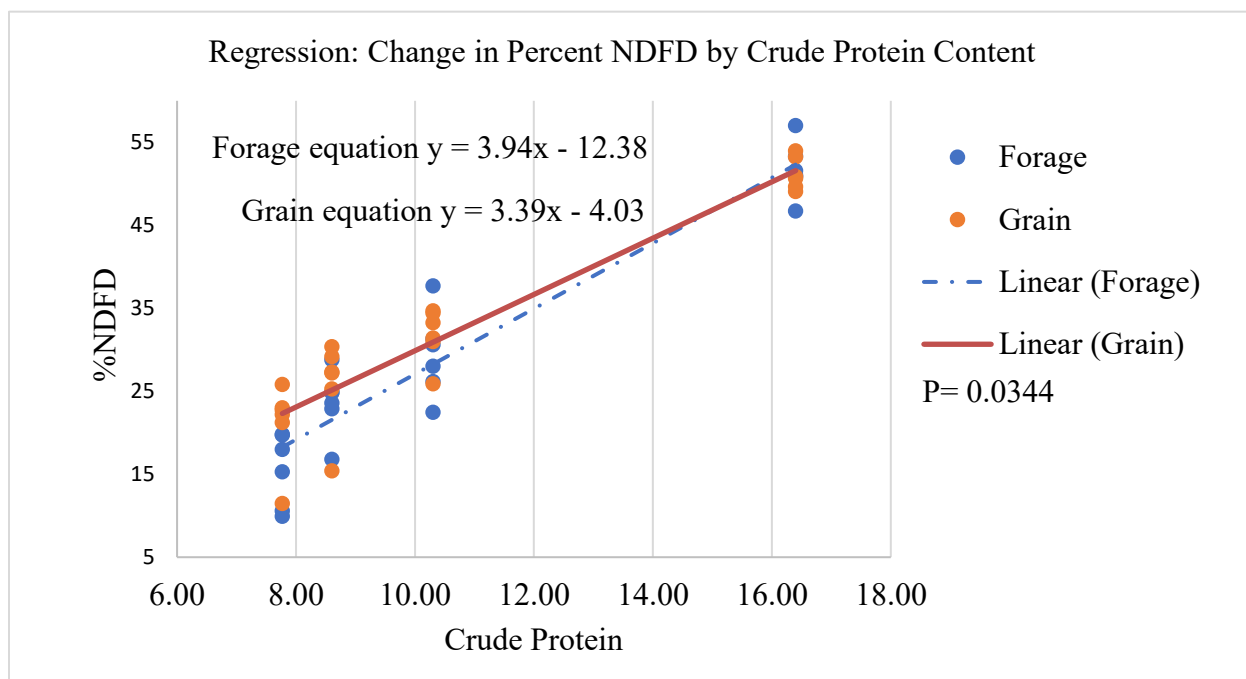


Figure 9. Diet of fecal inoculate donor by crude protein interaction in neutral detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

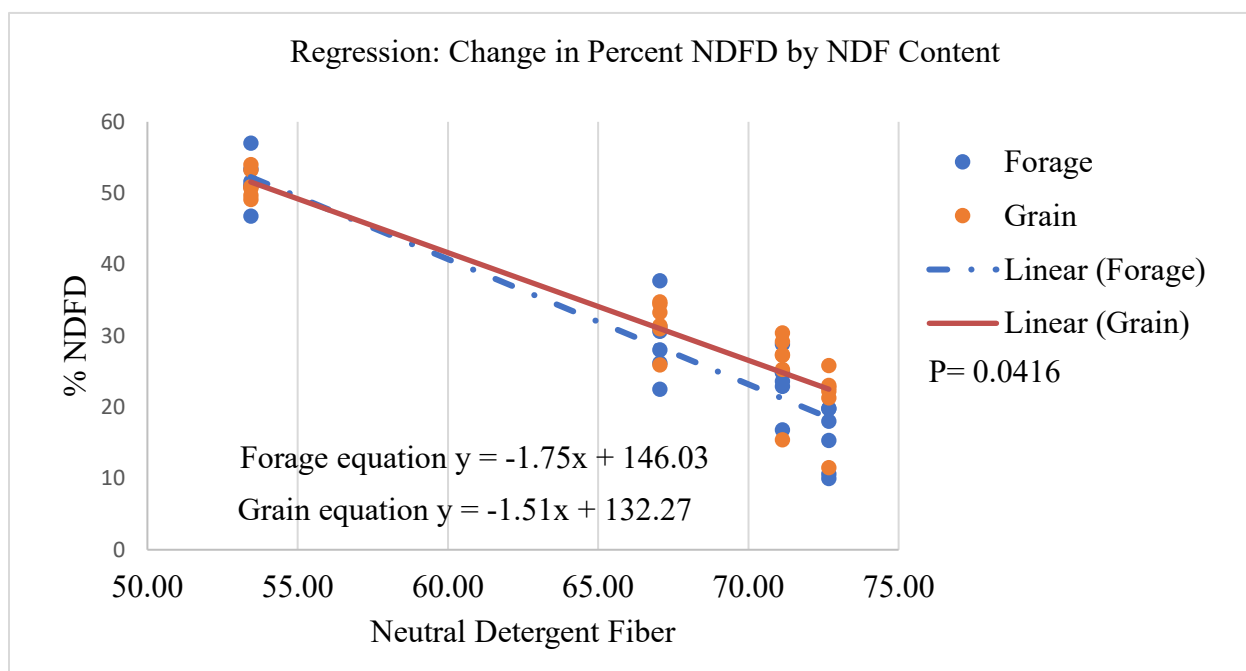


Figure 10. Diet of fecal inoculate donor by neutral detergent fiber interaction in neutral detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

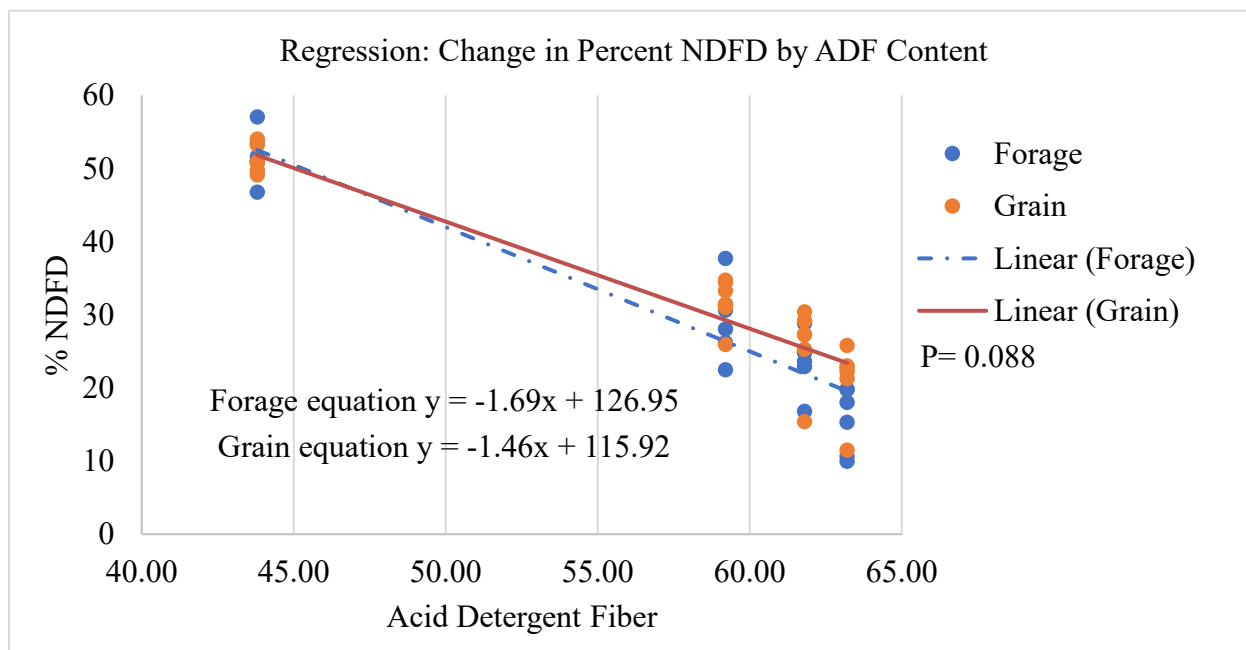


Figure 11. Diet of fecal inoculate donor by acid detergent fiber interaction in neutral detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

Table 9. Diet of fecal inoculate donor by crude protein, diet by neutral detergent fiber, and diet by acid detergent fiber regressions of dry matter digestibility analysis.

	Grain Diet		Forage Diet	
	Intercept	Slope	Intercept	Slope
CP	7.6±1.76	3.8±0.12	1.8±1.76	4.1±0.12
NDF	160.8±3.8	-1.7±0.05	168.2±3.8	-1.8±0.05
ADF	142.5±4.3	-1.6±0.07	148.4±4.3	-1.8±0.07

<sup>a,b</sup>Row means (±SEM) with different superscripts indicate significant differences in the slope (p<0.05). Forage parameters crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using wet chemistry and NIRs spectrometry. Digestibility parameters: dry matter digestibility (DMD) was analyzed using the Ankom Daisy<sup>II</sup> Incubator and Ankom<sup>2000</sup> Fiber Analyzer.

Table 10. Effect of diet by percent crude protein and diet by percent neutral detergent fiber in dietary forages on dry matter digestibility

	Grain Diet	Forage Diet	P-value
%CP			0.06
16	68.6±1.4	68.0±1.4	0.78
12	53.3±1.2	51.5±1.2	0.29
8	38.1±1.2	34.9±1.2	0.08
%NDF			0.06
72	38.6±1.2	35.5±1.2	0.08
63	53.9±1.2	52.1±1.2	0.3
54	69.1±1.4	68.6±1.4	0.8

<sup>a,b</sup>Row means (±SEM) with different superscripts indicate significant differences in the slope (p<0.05). P-values with an asterisk indicate significance (P<0.05). Forage parameters crude protein (CP) and neutral detergent fiber (NDF) were measured using wet chemistry and NIRs spectrometry. Dry matter digestibility (%DMD) was analyzed using the Ankom Daisy<sup>II</sup> Incubator.



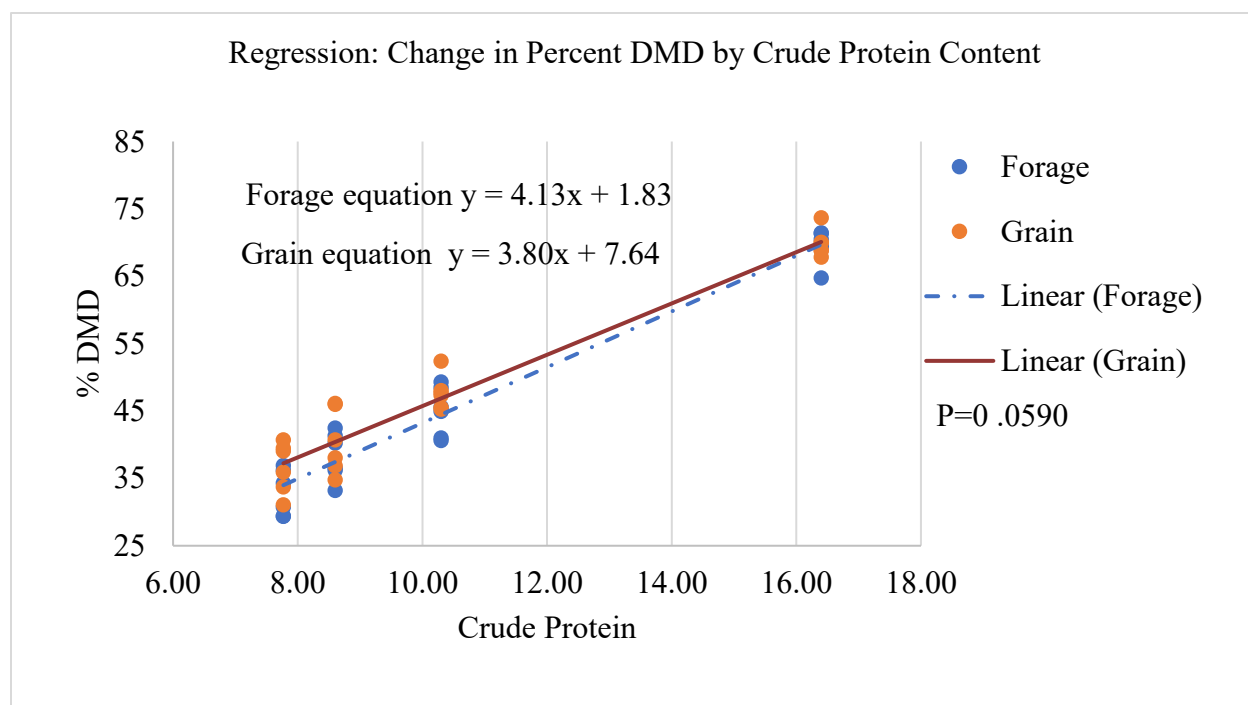


Figure 12. Diet of fecal inoculate donor by crude protein interaction in dry matter digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

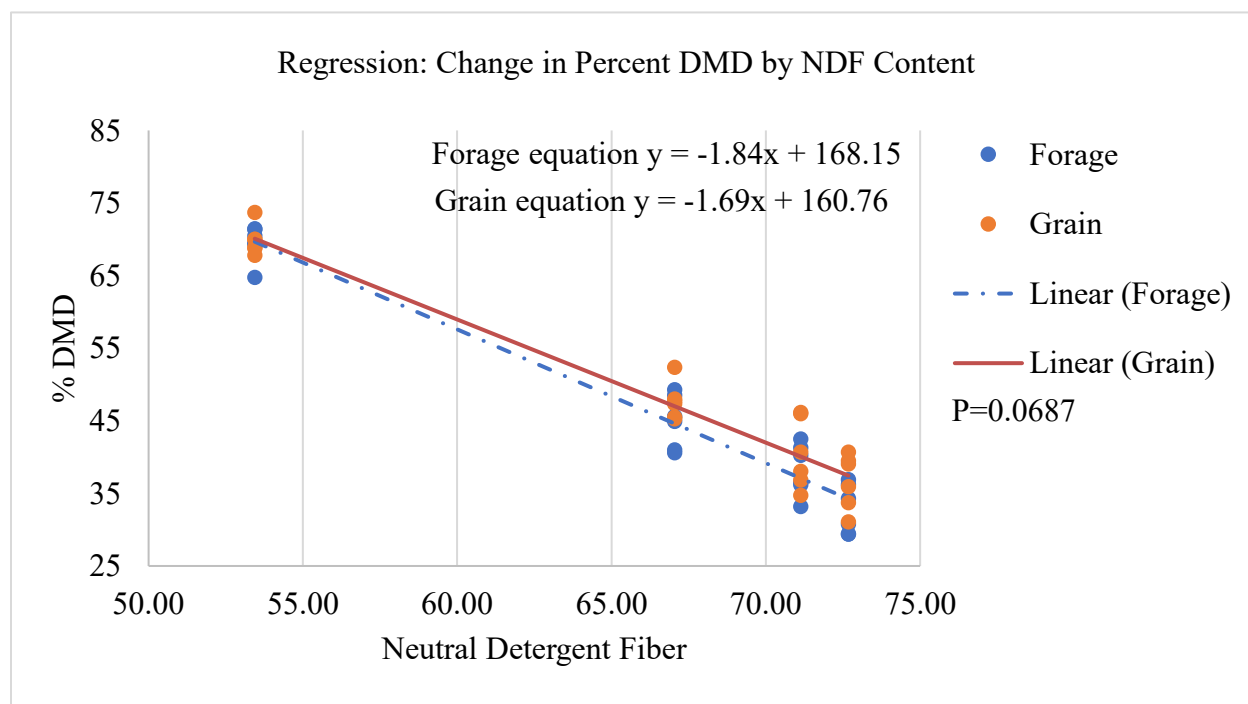


Figure 13. Diet of fecal inoculate donor by neutral detergent fiber interaction in dry matter digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

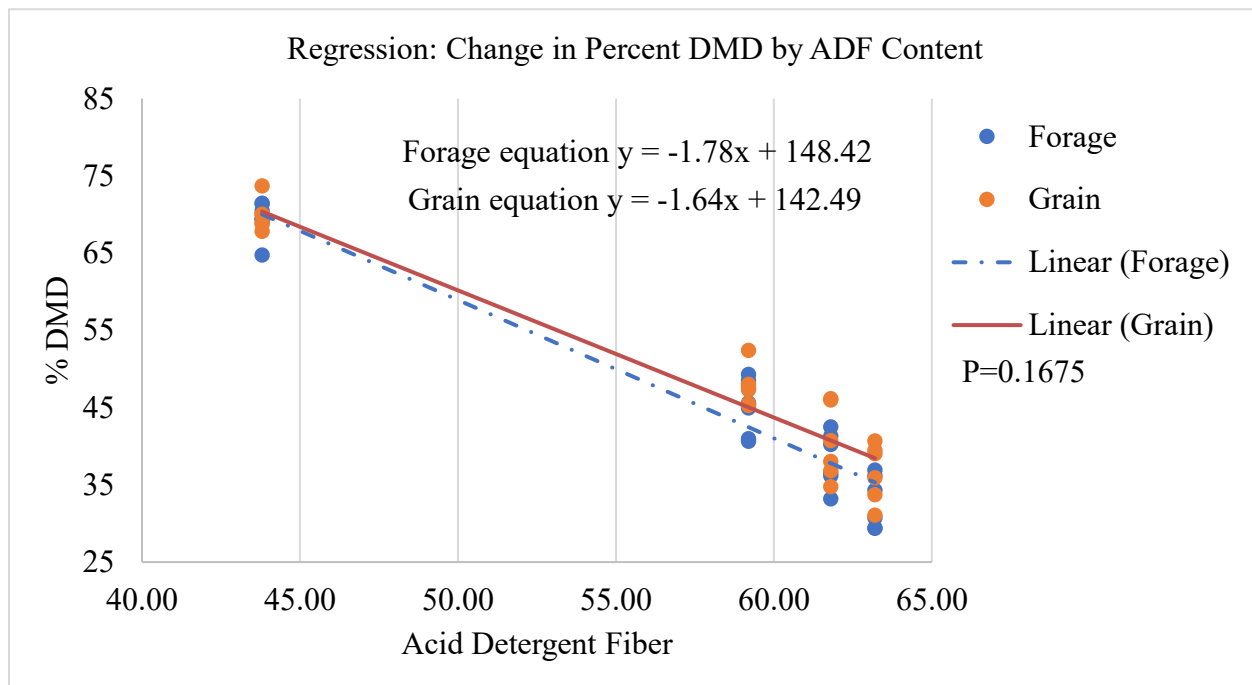


Figure 14. Diet of fecal inoculate donor by acid detergent fiber interaction in acid detergent fiber digestibility analysis of covariance including regression equations by diet of fecal inoculate donor.

## **DISCUSSION**

Significant horse variation was apparent across all DMD and NDFD analyses leading the author to suggest pooling fecal samples to inoculate in vitro digestion systems might improve accuracy. To the author's knowledge, no study has evaluated the efficacy of pooling fecal samples for inoculate preparation. As horse is a random factor, it may be useful to determine if pooling fecal samples provides a more accurate estimation of DMD for individuals and whether fecal donor diet impacts the estimation. Given that a foal's hindgut is inoculated during the first few days after birth through methods such as coprophagy, it is reasonable to believe the microbial population is constantly evolving to adapt to the environment within the individual. In addition, the diverse and conflicting reports of a core microbiome within the equine hindgut suggests there is high variability between individuals and potential redundancies in microbial function (Turnbaugh and Gordon, 2009). Based on the results of this study, pooled fecal samples may elucidate a more accurate estimation for the average equine. Conversely, pooling fecal samples may drive competition toward the inevitable proliferation of the most adapted species or symbiotic community of bacteria across all subjects, therefore overestimating digestibility of feedstuff in the average horse (Kristoffersen et al., 2016).

### **Digestibility**

Shifts in microbial populations within the equine hindgut due to dietary changes have been well established (Drogoul et al., 2001; Julliand et al., 2001; Milinovich et al., 2006; Daly et al., 2012; Harlow et al., 2015; Harlow et al., 2016). However, differences in feedstuff degradation resulting from the population changes have not been completely elucidated. When experiments were conducted in cattle, donor diet affected the digestibility of organic matter as a consequence of altering the fecal inoculate (Akhter et al., 1999). The present study yielded a

tendency for a grain diet to increase digestibility of dry matter dependent on forage parameters, CP and NDF ( $P=0.06$ ). Most notable differences in dry matter digestibility were seen in forages with  $CP \leq 8$  and  $NDF \geq 72$  ranges ( $P=0.08$ ). A significant increase in neutral detergent fiber digestibility was demonstrated as a result of a grain diet by forage parameters, CP and NDF ( $P=0.03$  and  $P=0.04$ , respectively). Again, the most noteworthy increases occurred in forages with  $CP \leq 8$  and  $NDF \geq 72$  ( $P=0.08$ ) where horses on a grain diet had a slight edge in digestibility over forage fed horses. In ruminants, DMD and NDFD have been positively correlated with forage CP content, and negatively correlated with NDF and ADF composition. Correlation coefficients suggested that high NDF and low CP were indicative of a low-quality forage (Du et al., 2016). The slopes of DMD and NDFD in relation to CP and NDF in the present study indicated the same was true for equine. However, as the forages declined in quality cracked corn may have a positive associative effect on NDFD and DMD.

Similar to the increase in digestibility of low-quality forages in the present study, addition of a concentrate component to rations increased digestibility of straw hay ( $CP=2.1$ ) revealing a positive association between low quality forages and concentrate (Kienzle et al., 2002). Authors postulated that the increase in fermentable carbohydrates and potentially the presence of available nitrogen in the cecum enhanced all microbial activity, including cellulolytic bacteria (Kienzle et al., 2002). Available nitrogen was thought to contribute to microbial activity due to the low crude protein content of the low-quality straw. However, in the present study, an isonitrogenous diet was fed, thus, the aforementioned assumption cannot be supported by the results of this study. Oldham (1984) described an increase in dry matter digestibility as a result of protein supplementation in cattle with those on a corn silage diet eliciting a stronger response compared to cattle on a grass silage diet. In this study however, the similarities in nitrogen concentration between diets suggested the increased digestibility was a result increased starch

reaching the hindgut, supplying sufficient energy substrates. A study by Nousiainen and associates (2009) also demonstrated positive associative effects increasing forage digestibility when substituting cereal grains for the fiber components of a ration. However, these were accompanied by negative effects such as increased rate of passage and lower digestibility of fiber found in concentrate. In a further study by Murray et al. (2003), as cited by Earing et al. (2010), feedstuff digested with inoculum collected from ponies fed a concentrate diet was digested more than fecal inoculum from ponies on a forage diet.

In ruminants, improved conditions for cellulolytic populations have been demonstrated when starch is replaced with a fibrous component in a ration, effectively increasing the NDFD (Nousiainen et al., 2009). An equation has been developed to estimate NDFD suggesting for every 1 percent of starch incorporated in a diet, NDFD decreases by 0.59 percent, developed on the estimation of ruminal pH decline, creating an unaccommodating environment for fibrolytic bacteria. In this study, however, NDFD is increased with the addition of starch in the diet (de Souza et al., 2018).

In contrast to the results in the present study, Earing et al. (2010) exhibited no differences in the in vitro digestibility of DM or NDF of feedstuff when horses were fed different diets. Among feedstuff tested was a timothy hay diet, alfalfa diet, timothy hay: oat diet, and alfalfa: oat diet with mixed rations fed at 70:30 ratio. Although a significant increase in oat DMD was measured using the forage only inoculate at 30h, diets were not different at 48h or 72h. However, the author acknowledges the limitations accompanying the small portion of oats in the diet and lack of evaluation of high concentrate diets. As previously mentioned, small levels of starch are readily digested by amylase and absorbed in the small intestine. Depending upon total ration size, as a determining factor of rate of passage, and starch availability in oats, a mere 30 percent of the ration in oats may or may not be sufficient to overload the foregut's capacity for pre-cecal

starch digestion. If maintained under the limitations of foregut capacity, substantial amounts of readily fermentable starch would not reach the hindgut. Therefore, the potential for fecal inoculates to digest feedstuff similarly is retained. Furthermore, the different starch sources between the aforementioned and present study have previously exhibited contrasting effects on the hindgut microflora (Harlow et al., 2015; Harlow et al., 2016).

Corn has been implemented as eliciting the most volatile bacterial response, causing the predominant amylolytic bacteria to rapidly fester producing high concentrations of lactate, thus lowering pH beyond the threshold of optimal growth for neighboring microbes (Harlow et al., 2016). Alternatively, introduction of an oat diet containing an equivocal amount of starch yielded an environment with mixed dominance and more lactate utilizing bacteria, therefore attenuating the hostile environment created by *Streptococcus* spp. and *Lactobacillus* spp. (Harlow et al., 2016). The present study utilized cracked corn as the starch source, which provided more distinguishable results.

Increasing hydrolysable carbohydrates in the hindgut in the form of starch offers a quick energy source for many bacteria, including cellulolytic as mentioned by Kienzle et al. (2002). Another potential mechanism is starch increasing the activity of commensal bacteria such as *Treponema* spp. that aids in the attachment of *Fibrobacter succinogenes* to metabolize discarded xylans (Kristoffersen et al., 2016). Unfortunately, as pH measurements, bacterial enumerations, and genetic sequencing were not performed, only assumptions can be made in the present study.

## **Limitations**

The small sample size present in this trial inclines data to skew with slight experimental error or environmental factor. Narrow ranges of forage parameters limited the study from elucidating significant results. Larger sample size and a broader spectrum of forage parameters

could lead to a more robust study. Measuring pH, performing bacterial enumerations, and genetic sequencing are imperative to reveal changes in bacterial communities occurring throughout the in vitro digestion system. Understanding the shift in the bacterial population could deliver valuable knowledge regarding starch utilization in the equine hindgut.

## CONCLUSIONS

As expected, there was significant variation between individual horses accounting for a sizeable portion of the diversity in this trial. In agreement with previous studies, ADFD was not influenced by diet x forage parameters. Evaluation of NDFD and DMD revealed a trend for grain diets to increase fiber digestibility in low quality forages that contained no more than 8 percent crude protein and at least 72 percent NDF. Differences in the slope of neutral detergent fiber digestibility were significant among crude protein and neutral detergent fiber forage parameters. However, the digestibility slopes of diets within dry matter digestibility failed to distinguish significant results, although trends were seen in NDF x diet and CP x diet interactions. Previous studies reveal controversial results regarding a grain diet's effect on digestibility of fiber. In this study, grain increased the digestibility of lower quality forages.

In this trial, increased digestibility of low protein forages on the grain diet was not a result of increased nitrogen as crude protein concentrations were similar between diets. The author of this study suggests the increase in high energy feedstuff reaching the hindgut led to an increase in microbial activity therefore increasing fermentation of low-quality fiber. In addition, digestibility values of forages with lower NDF and higher CP, often associated with higher-quality forage, were grouped closely between diets averaging within one percent DMD and NDFD. Similar digestibility of high-quality forage between diets may indicate the more readily available nutrients, therefore, a highly active microbial population is not necessary.

Few studies have focused on the microbial population's ability to digest feedstuff following a change in diet. With a more robust study, further results may be elucidated. Lack of bacterial analysis and parameters therein prohibit further investigation into the mechanism



behind the observed trends. Results of the present study indicate a difference in the digestibility slope across forages by diet. However, only assumptions can be made as to the reason behind the apparent trend for grain to increase the digestibility of fiber as bacterial enumerations were not conducted due to financial constraints. Further studies should be conducted utilizing next generation sequencing in the analysis in order to characterize the ephemeral changes that occur in the hindgut.

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