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In Vitro Digestibility of Forages Incubated with Equine Fecal Inoculum Stored Under Varying Conditions

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IN VITRO DIGESTIBILITY OF FORAGES INCUBATED WITH EQUINE FECAL INOCULUM STORED UNDER VARYING CONDITIONS

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Shianne Walther

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ABSTRACT

This study evaluated the *in vitro* effect on digestibility of forages when incubated with microbial inoculum formed from equine feces stored under varying conditions. Fecal material was stored at four storage conditions of 12˚C for 6 hours, 12˚C for 12 hours, 23˚C for 6 hours and 23˚C for 12 hours under aerobic conditions. Stored fecal material was used to form microbial inoculum for use in an ANKOM Daisy II Incubator. Nine different forage samples were digested in the ANKOM Daisy II Incubator to evaluate interactions between storage condition of microbial inoculum and chemical composition of digested forage samples. Forage samples chosen covered a wide range of crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) values to determine if forage quality had an effect. Dry matter digestibility (DMD) was determined after incubation. An ordinary least squares (OLS) regression was used to analyze variation among storage conditions, forage samples, and replicates. Significant differences were observed among forages and replicates ($P<0.05$). No differences ($P>0.05$) were observed among the interactions between storage conditions and forage samples. Results show fecal material stored at 12˚C or 23˚C for 6 or 12 hours provides no difference when used for the formation of microbial inoculum. Further research is needed to determine how the microbial population within a fecal sample change when exposed to aerobic conditions for varying amounts of temperature and time.

KEYWORDS: equine, *in vitro* digestion, microbial digestion, Daisy II incubator, fecal inoculum, forage digestion

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INTRODUCTION

Need for the Study

The intestinal tract of the horse is home to a vast, diverse, and poorly understood microbial population (Blikslager et. al., 2017). Gut microbes support the host by providing nutrition, metabolism, and a wide range of other functions supporting overall health of the horse. Hindgut microbiotas are composed primarily of a core group of bacteria that adapt in response to dietary changes. While a change in forage type has no significant long-term change in hindgut population, an abrupt change from an all-forage diet to a diet high in starch or fat has been shown to decrease the core bacteria population (Julliand and Grimmn, 2017). By gaining knowledge of the evolution of microbes in the hindgut researchers are better able to formulate healthy diets to decrease gastrointestinal disturbances.

The types of digestibility studies conducted include *in vivo* and *in vitro* trials. *In vivo* studies require either total collection of all fecal material excreted over the research period, a fistulated subject, or for the subject to be euthanized and dissected. While these studies give researchers a more accurate look at the hindgut microbial population cannulation is a permanent procedure that is expensive and must be maintained for the rest of the horse's life. Euthanasia allows researchers to collect contents from various portions of the equine hindgut not normally accessible for collection. Euthanizing horses for research requires special permission of the owner, can be costly and equines are not able to be studied under different types of diets. *In vitro* digestibility trials are less invasive and therefore more ethical and cost effective when compared to *in vivo* studies. Most importantly, they provide a faster, more accessible way of evaluating digestibility and allow for multiple forage samples to be tested at one time.

Previous studies have proven equine fecal material is an acceptable form of microbial inoculum for digestive studies (Lowman et. al., 1999). A lack of research exists on how time and storage temperature changes inoculum viability. A study by O' Donnell suggested if fecal material was chilled or frozen it was no longer viable (O'Donnell et. al., 2020). Further research on preserving fecal material at less severe temperatures and for a longer period of time may help researchers to better preserve fecal inoculum in order to gather samples further distances from laboratory facilities.

Objectives

The objective of this study was to determine the effects of storage condition on the viability of microbial inoculum on dry matter digestibility (DMD) when the feces were stored under aerobic conditions at 12˚C and 23˚C for 6 and 12 hours. In addition, it is also imperative to assess any interactions between storage conditions of microbial inoculum and various forage chemical compositions of DMD, acid detergent fiber (ADF), and neutral detergent fiber (NDF).

Null Hypothesis

The null hypothesis of the present study is that equine fecal material stored at temperatures of 12˚C and 23˚C and times of 6 hours and 12 hours used for microbial inoculum does not have an effect on estimates of DMD.

LITERATURE REVIEW

Equine Digestion

The equine gastrointestinal tract is divided into two sections by function and common purpose into the foregut, encompassing the mouth, esophagus, stomach, and small intestine and the hindgut, containing the cecum, colon, and rectum. Equines are monogastric herbivores referred to as hindgut fermenters. The hindgut makes up 53% of the equine digestive tract and is devoted to microbial fermentation and forage digestion (Jones, 2019). Digesta pass relatively quickly (5 hours on average) through the stomach and small intestine where contents undergo enzymatic digestion. After passing through the foregut, digesta spend an extended amount of time (35 hours on average) in the hindgut to undergo an intense microbial fermentation process (Van Weyenberg et. al., 2006).

When allowed to graze free choice horses select for succulent forages high in water, soluble proteins, lipids, sugars, and structural carbohydrates. Today, horses are often stalled and restricted to a diet high in dried forages and supplemented with high energy feedstuffs, in the form of high-starch cereal grains or high-fat feeds, like oils (Frape, 2008). A significant change in diet also leads to a change in the hindgut microbial population (Daly et. al., 2012).

Starting with the beginning of the digestive system the five main parts of the mouth include the lips, teeth, salivary glands, tongue, and jaws. Prehensile lips allow for selective consumption of forages sorting less desirable forages aside in order to obtain more palatable forages. The teeth, tongue, and mandible are responsible for mastication by biting, grinding, and mixing feedstuffs with mucosa produced by the salivary glands within the mouth. An intensive mastication process allows food to be chewed thoroughly decreasing forage particles to less than 1.6 mm in length before swallowing (Frape, 2008). Physical presence of feed stimulates the

secretion of saliva by the paired parotid, submandibular, and sublingual glands (Al-Sobayil et. al., 2008). Saliva itself contains two major types of protein secretion: a serous secretion containing a-amylase, an enzyme for digesting starches, and a mucus secretion containing mucin for lubrication purposes (Guyton, 1991). Unlike ruminants, saliva in horses has no notable enzymatic function. Salivary glands secrete 10-12 L of saliva per day to lubricate the passage of digesta and buffer the proximal region of the stomach. Once the horse swallows, peristaltic waves push the bolus from the mouth to the esophagus to be deposited in the stomach.

The junction between the esophagus and stomach is guarded by a strong muscular opening known as the cardiac sphincter. This muscular opening allows feed to be ingested but restricts regurgitation even under painful abdominal stress. The stomach is approximately 10% of the gastrointestinal (GI) tract holding 8-18 L of material. Horses have a simple stomach divided into non-glandular and glandular regions. The non-glandular region makes up the proximal region of the stomach and is lined with stratified squamous epithelium cells, much like the esophagus, that are sensitive to stomach acid. Non-glandular and glandular regions overlap creating a folded border called the margo plicatus. Microscopically, it is identified as an abrupt transition within the lamina epithelialis from non-keratinized stratified squamous epithelium to a simple columnar epithelium.

The glandular region of the stomach is further divided into three sectors: proper gastric, cardiac, and pyloric. Glandular regions are made of a collection of secretory cells that include parietal, chief, mucous neck, and enteroendocrine cells. Parietal cells secrete hydrochloric acid (HCL) and intrinsic factor. Secretion of HCL is continuous resulting in a highly acidic environment between 1.8 to 3.5 pH (Guyton, 1991). Chief cells secrete the proenzyme pepsinogen, which is converted to the active enzyme, pepsin, when mixed with HCL. In this

process the pepsinogen molecule is split to form the pepsin molecule. Mucous neck cells secrete a thin acidic mucus that allows easy slippage along the gastrointestinal tract while also preventing chemical damage to the epithelium (Guyton, 1991). Enteroendocrine cells secrete various hormones including gastrin, ghrelin, histamine, serotonin, and somatostatin.

Chyme moves from the stomach to the small intestine via the pyloric sphincter. The small intestine makes up approximately 30% of the equine digestive tract, is 21–25 m in length, and can hold 57 L of material. The small intestine is further divided into three segments: the duodenum, jejunum, and ileum. Chemical digestion primarily takes place in the small intestine. Most of digestion and absorption occur in the duodenum and jejunum, protecting the ileum from a high nutrient load. The absorptive surface of the intestinal mucosa contains many folds called valvulae conniventes which increase the surface area of the absorptive mucosa by threefold (Guyton, 1991). Villi line the entire surface of the small intestine and project approximately one millimeter from the surface of the mucosa. These villi increase the absorptive surface by tenfold. Microvilli are fingerlike projections located on the villi, referred to as the brush border that contain digestive enzymes and increase the surface area of the small intestine by another 20-fold. Crypts of Lieberkühn are pits located at the base of villi that secrete an extracellular fluid that maintains chyme in a fluid state (Blikslager et. al., 2017).

The presence of nutrients such as proteins, lipids, and carbohydrates in the duodenum stimulate endocrine cells to release cholecystokinin (CCK). This in turn stimulates the pancreas to secrete digestive enzymes. The secretion of CCK also stimulates the release of bicarbonate from the pancreas to neutralize acidity coming from chyme in the stomach. Protein digestion in the small intestine begins with chymotrypsin and trypsinogen being released from the pancreas. Trypsinogen is converted to its active form, trypsin, in the presence of enterokinase secreted by

brush border enzymes. Trypsin activates other enzymes known as proteases to further digest proteins. Together, these enzymes break proteins down to tripeptides, dipeptides, and individual amino acids (Callahan et. al., 2020). Within the enterocyte, peptidases are hydrolyzed into constituent amino acids which move passively into the blood system. Fats are emulsified by bile salts from the liver, making them more accessible to digestive enzymes by increasing the surface area for them to act. Triglycerides are broken down by pancreatic lipase into fatty acids, monoglycerides, and some free glycerol. Bile salts then cluster around the products of fat digestion to form micelles, which aid in absorption. Once in the intestinal cell, short and medium chain fatty acids and glycerol can be directly absorbed into the bloodstream (Callahan et. al., 2020). Carbohydrate digestion and absorption in the small intestine begins by digestible dietary carbohydrates being hydrolyzed by pancreatic a-amylase and brush border membrane disaccharides, sucrase, maltase, and lactase. The monosaccharides produced by brush border hydrolysis such as D-glucose, D-fructose, and D-galactose are then absorbed across the enterocyte brush border membrane by specific transporters (Dyer et. al., 2002).

The ileocecal junction marks where the foregut ends and the hindgut begins. The hindgut consists of the cecum, colon, rectum. The hindgut is an anerobic environment where microorganisms ferment hydrolysable nutrients that escape digestion and absorption in the proximal gastrointestinal tract as well as indigestible complex molecules. In total, the hindgut of the horse can hold 23.8 L (Julliand and Grimm, 2016).

The cecum is a comma shaped organ that is approximately 1 meter long and can hold 16–68 liter worth of material (Ross and Hanson, 1992). This large range in cecum size is due to the age and weight of the animal. The cecum functions similarly to the rumen in ruminants. When compared to cattle, horses are much less efficient at utilizing nutrients produced by

microbes due to gastrointestinal anatomy. In ruminants, the rumen precedes the small intestine allowing for a greater absorption of nutrients. In equines the cecum succeeds the small intestine therefore the large intestine is responsible for the absorption of all nutrients produced by microbial fermentation.

The cecum is comprised the base, body, and apex. The cecal base curves ventrally into the cecal body. The cecal body has four haustral folds, or longitudinal bands that create deep sacculation's and ends in a cul-de-sac apex. The base of the cecum is divided into cranial and caudal portions by a transverse fold starting at the floor of the cecal base and rising just below the ileocecal orifice (Ross and Hanson, 1992). Coordinated cecal contractions consist of haustra alternately contracting and expanding. Various motility patterns allow for adequate mixing of the intestinal contents with mucosa and microbes while also providing adequate time for microbial attachment and fermentation to take place. The cecal artery, originating from the ileocolic artery, supplies blood to the cecum. Cecal arteries and veins are affixed by a loose mesenteric attachment along cecal bands.

Ingesta and gas move through the cecocolic orifice into the colon. The colon is further divided into the large colon, transverse colon, and small colon. The large colon is 4 m in length and can hold approximately 80 L of ingesta (Blikslager et. al., 2017). The large colon is comprised of the right ventral colon (RVC) , the left ventral colon (LVC), the left dorsal colon (LDC), and the right dorsal colon (RDC). The pelvic flexure is located between LVC and LDC. This abrupt change in direction delays emptying of the ventral colon. The large colon forms a long U-shaped loop that is attached to the dorsal body wall at the RDC and RVC. The RVC and LVC are sacculated by four bands. The presence of bands fades at the pelvic flexure leaving the LDC and RDC moderately sacculated. Ventral and dorsal segments are attached to the body by

intercolonic mesentery however, this U-shaped loop is still relatively mobile and prone to anatomic displacements and physical obstructions where the lumen narrows.

The transverse colon joins the RDC and the small colon (SC). The SC is approximately 4 m long, 6–8 cm in diameter (Ross and Hanson, 1992) and holds approximately 18–19 L of ingesta (Lewis, 1995). Wide, coarse sacculations are created by muscular mesenteric and antimesenteric bands. The SC is where fecal balls are formed. The caudal mesenteric artery is the main blood supply for the SC branching into the left colic artery, and the cranial rectal artery. The digestive system ends with the rectum followed by the anus where feces are excreted.

The Hindgut Microbiome

Without the hindgut microorganisms there would be no horse! The community of microbiota that colonize the GI tract consist of microbial organisms, including protozoa, bacteria, fungi, bacteriophages, and archaea (Bustamante et. al., 2021). Cell wall carbohydrates, which include cellulose, hemicellulose, and pectin, represent 35-60% of the typical equine diet (Julliand and Grimm, 2016). These cell wall components escape digestion by the hydrolytic enzymes of the foregut and can only be broken down by microbial enzymes of the hindgut due to the structure of the cell wall. The hindgut of the horse is an ideal environment for microorganisms promoting growth and fermentative activity. Approximately 10-40% of starch digestion also occurs in the hindgut of the horse. This is due to the structure of a starch granule and the limited capacity of amylase or fructanase in the small intestine (Julliand and Grimm, 2016). Each compartment of the hindgut encompasses its own ecosystem unique in biological and environmental components. Microbial communities within the microbiome are characterized by microbial diversity, structure, metabolic activities, and resulting end products. Hindgut plant

carbohydrate degradation begins with the hydrolysis of complex polysaccharides and concludes with the fermentation of simple sugars. Microbial utilization is crucial as it converts indigestible plant materials into VFA's that can be absorbed and used as energy. (Julliand and Grimm, 2016). Despite the importance of the microbes supplying energy, limited information is known about the overall composition of the microbiome.

Protozoa were the first microbe to be discovered in the hindgut of the horse due to their large size. Protozoa are free-living unicellular eukaryotes that carry out complex metabolic activities. Concentration varies from 10^3–10^6 cells/mL of cecal or colonic content with concentrations being lower in the cecum than in the colon (Kern et. al., 1973,1974; Goodson et. al., 1988; Moore and Dehority, 1993). Four classes of protozoa have been identified including *rhizopoda*, *mastigophora*, *cilata*, and *suctorial*. Ciliates are the most predominant with roughly 30 genera encompassing approximately 50 species throughout the hindgut of the horse (Grimm et. al., 2016). Protozoa play a very minor role in fiber breakdown. While cells belonging to the genus *Cycloposthimum* provide some contribution to plant fiber breakdown. (Moore and Dehority, 1993), removed protozoa from the hindgut of the pony and only saw a slight decrease in overall DMD with no effect on cellulose digestion (Julliand and Grimm, 2016).

Bacteria are single celled microorganisms belonging to the prokaryotic group of cells, meaning they do not have a true nucleus. *Bacterium Intestinals Equi* or bacteria were first studied in 1894 by Dylar and Keith. A major limitation for research at the time was the lack of anerobic conditions available. Today, it is understood 38 (Kern et. al., 1973) to 80% (Kern et. al., 1974) of bacteria that live in the hindgut are sensitive to oxygen. Total anerobic bacteria varies from 10^7–10^{11} cells/L of cecal or colonic content (Sadet-Bourgeateau et. al., 2014). The bacterial community represents the majority of hindgut microbiota (Julliand and Grimm, 2016).

More than 20 different types of phyla are present in the equine hindgut however the dominant five are: *firmicutes, bacteroidetes, proteobacteria, actinobacteria*, and *verrucomicrobia*. Depending on biology and methodology proportions of *bacteroidetes* and *verrucomicrobia* vary. Regardless of the method, *firmicutes* are the most abundant phyla of bacteria in horses (Blikslager et. al., 2017). The bacterial community in the proximal and distal parts of the hindgut differ with the change in population occurring at the pelvic flexure (Hastie et. al., 2008; Dougal et. al., 2012; Sadet-Bourgeateau et. al., 2014). Bacteria play a large role in the breakdown of plant fiber. Hindgut bacteria can be grouped by function, which include but are not limited to starch-utilizing bacteria, lactate-utilizing bacteria, cellulolytic, and hemicellulolytic. Cellulolytic bacteria make up 1–9% of the total bacteria in the cecum (Kern et. al., 1974; Julliand, 1996). Some bacteria colonize the liquid medium while others adhere to plant fragments.

Anerobic fungi are multicellular eukaryotic organisms that are heterotrophic and play an important role in nutrient cycling. Fungi convert feed to protein with fiber degrading enzymes and invasive growth (Hess et. al., 2020). Fungi were first discovered in 1910, but were mistaken for protozoa. Fungal zoospores were not reported in the equine hindgut until 1981 (Juliand and Grimm, 2016). Anaerobic fungi are unique to the fungal kingdom in that they possess hydrogenosomes instead of mitochondria. Hydrogenosomes metabolize glucose into cellular energy without the presence of oxygen. Researchers predict there are at least 34 fungal genera and over 250 species in existence (Edwards, 2019). Fungi reproduce asexually cycling between flagellated zoospores and sporangia (Heath et. al., 1983). There are approximately 3.2-4.7 $\times 10^4$ zoospores/ml of cecal contents (Orpin, 1981). Fungi were quantified using the 5.8S rRNA gene and showed there is no significant difference between gut regions or animals (Dougal et. al., 2012) Three fungal species have been reported : *Piromyces equi*, *Piromyces citronii*, and

caecomyces eqi. Anerobic fungi play a big role in DMD and increase digestion by 7-9% (Gordon and Phillips, 1993). Further research of fungi is needed to quantify their contribution to fiber digestion.

Bacteriophages are viruses that infect and replicate within bacteria and archaea. The term is derived from "bacteria" and the Greek word phagein meaning "to devour". They were first discovered using electron microscopy in the cecum and colon (Alexander et. al., 1970). No difference has been seen in concentration between the various regions of the hindgut. Some of the dominant bacteriophages include *siphoviridae, myoviridae, podoviridae,* and a *vertebrae Orthopoxvirus sp*. (Cann et. al., 2005). Fecal phage particles measure to be approximately 10^{10}–10^{11} cells/g of fecal material. Bacteriophages are free or in association with bacteria. This indicates they may play a role in regulating bacteria species distribution in the hindgut. The exact role of bacteriophages is still unknown today (Juliand and Grimm, 2016).

Archaea were discovered in the equine cecum in 1996. They are a group of micro-organisms similar but different in structure than bacteria. Researchers have measured there are 10^4–10^6 cells/g of the prokaryotic methanogen community in wet weight of equine cecal contents (Morvan et. al., 1996). Methanogen's archaea are found to have greater concentration in the right dorsal colon than in the cecum. When isolated from the horse's hindgut these methanogens use H_2 and CO_2 to produce methane. In the ruminant animal, methanogenic bacteria boost the carbohydrate degrading activity of cellulolytic bacteria (Julliand and Grimm, 2016). No study has focused specifically on the methanogenic archaea in the hindgut of the horse.

The deterioration of complex polysaccharides begins with the attachment of microorganisms to plant cell walls. Microorganisms primarily responsible for plant cell wall

degradation are the fungal species of *piromyces genus* and bacterial species of the *ruminococcus* and *fibrobacter genera* (Jouany et. al.,2009). Microorganisms responsible for amylolytic degradation are the bacterial species *streptococcus* and *lactobacillus* genera with isolated strains being *streptococcus bovis, S. equinus, lactobacillus salivarius, and L. mucosae* (Kern et. al., 1973). Adhesion of these microorganisms concentrates bacterial enzymes to substrates for the hydrolyzation of polysaccharides.

The main products of carbohydrate digestion include short chain volatile fatty acids, mainly including acetic, propionic, and butyric acids (Blikslager et. al., 2017). These VFA's are easily absorbed by mucosa. Increased activity of fibrolytic microorganisms induce high levels of acetic and moderate levels of butyric acids. Increased activity of amylolytic microorganisms induces an increase in propionic acids. Therefore, fibrolytic activity from plant cell wall fermentation can be evaluated in the ratio [(acetate + butyrate)/propionate]. Small amounts of lactic acid are also produced but are poorly absorbed and therefore do not serve as a major nutrient. If large amounts of lactic acid are produced it will lead to intraluminal osmolality (Argenzio et. al., 1975). Composition of VFA's is ultimately determined by the nature of the plant carbohydrates and microbiota type.

Effect of Diet on Microbial Population

The equine diet has a significant impact on the microbiome of the hindgut. Feed components that go undigested by the foregut pass to the hindgut and deliver growth-promoting or growth-inhibiting factors that guide the equilibrium of the microbial community. Non-digestible plant cell walls provide a medium for the growth of fibrolytic microorganisms which in turn provide energy via the end products of their metabolism (Julliand and Grimm, 2016).

Forage based diets tend to have a very diverse community of microbes making the digestive system less susceptible to imbalance and more resilient to change. In a concentrate-based diet bacterial richness and diversity are lowered and starch has the ability to cause a decrease in resilience and a greater chance of dysbiosis (Julliand and Grimm, 2017). Studies have shown that dysbiosis in the hindgut can lead to serious equine health issues such as colic and laminitis (Cohen et. al., 1995; Reeves et. al., 1996; Tinker et. al., 1997; Hudson et. al., 2001). This imbalance is often caused by an abrupt change in diet hindgut microflora are not equipped to handle.

An abrupt increase of soluble carbohydrates in the diet leads to an increase in rate of micro-organism multiplication. This increase in micro-organisms results in an increase in concentration as well as a modification of metabolism for some micro-organisms. This then leads to an increase in total VFA's and lactate. Because of this change the digestive ecosystem's buffering system becomes saturated, thus dropping pH. This decrease modifies characteristics of the microbial ecosystem by decreasing cellulolytic bacteria and increasing acidophyle flora. The decrease in cellulolytic bacteria thus decreases acetate while the increase in acidophyle flora increases lactate and propionate. This modification of the fermentative profile decreases pH even more thus repeating the cycle. (De Fombelle et. al., 2001).

An abrupt change from one forage type to another, with similar botanical and chemical composition, had no significant impact on cellulolytic, xylanolytic, or pectinolytic bacteria (Julliand and Grimm, 2017). An abrupt change from a high forage ration to a high concentrate ration has shown to significantly impact microbiota. Research done by Goodson studied an abrupt change from a 100% alfalfa diet to a diet composted of 86.7% ground corn and 13.33% soybean meal resulted in a significant increase of amylolytic bacteria within 24 hours (Goodson

et. al., 1988). A study by De Fombelle added barley to a fiber-based diet and reported an increase in *lactobacilli* and *streptococci* concentrations as well as an increase in lactate concentration within the first 5 hours (De Fombelle et. al., 2001). Twenty-nine hours after the diet change, no significant change in cellulolytic bacterial concentrations were reported. However, within the first few days, percentages of *xylanolytic* and *pectinolytic* bacteria were reported to decrease significantly. The increase of amylolytic activity and decrease of fibrolytic activity were consistent with the decrease of the ratio [(acetate + butyrate)/propionate] measured in the proximal hindgut. De Fombelle reported a greater variation in microorganisms in the right ventral colon than the cecum (De Fombelle et. al., 2001). This can be due to the fact that it is the first segment affected by overfeeding barley. This may also explain why the right ventral colon is a major site affected during colic. Forty-eight hours after an abrupt increase in concentrates, Goodson reported a large increase of anaerobic bacteria, a decrease in protozoan numbers, and a decrease in amylolytic numbers (Goodson et. al., 1988). After seven days, the hindgut microbial population leveled out and did not show differences between the diets (De Fombelle et. al., 2001).

Methods of Estimating Digestibility

The digestion of nutrients by the horse can be determined using either *in vivo* or *in vitro* methods. *In vivo* referring to a process performed or taking place in a living organism and *in vitro* referring to a process performed or taking place in a test tube, culture dish, or elsewhere outside a living organism. Popular *in vivo* methods include the total collection of feces, studying a canulated animal, and marker methods. With all digestibility studies, animals should be

allowed 7–21 days to adjust to the feed being offered before collecting samples for analysis (Zewdie, 2019).

The total collection method is considered to be the most accurate form of studying equine digestibility. Horses are individually placed in either a digestibility stall or a regular stall equipped with a fecal collection device. The goal for researchers is to completely recover all feces. The experimentation diet is fed to each animal in known quantities while refusals and fecal output are kept and recorded. Typically, a representative sample of 10% daily output from the feces is retained, dried and ground for chemical analysis (Zewdie, 2019). Studies estimating nitrogen balance will also collect and measure urine output. While the total collection method is the most accurate it also comes with difficulties. This method can bring about discomfort for the animals used as they are forced to stay in a relatively small space alone. This collection method cannot be used with nervous or working horses (Bergero et. al., 2005). Total fecal collection is also incredibly labor intensive. A study by Takagi collected feces bags every four hours during the day and once at night for a period of ten days per forage sample with a total of six samples (Takagi et. al., 2002). When considering using several animals with several fecal samples a significant amount of labor hours are required which come at a cost.

An easier alternative to the total collection method is the marker technique. This method merely analyzes a sample of feces rather than collecting the total feces. This method uses an indigestible reference substance added to the animal's diet. These indicators can be a natural constituent of the feed or added to the feed. Natural constituents of feed include acid-insoluble ash and indigestible ADF. Substances commonly added to feed include ferric oxide, lignin, silica, chromogen (Zewdie, 2019). The marker substance used must be completely non-absorbable and cannot affect the gastrointestinal tract or microbial population. The marker used

must be similar to the feed material and does not interfere with the digestive analyses. Digestion is calculated by the change in ratio of each nutrient with reference to the indigestible substance in the feed and feces (Zewdie, 2019). The formula for calculating the digestion coefficient of a nutrient is as follows:

$$\text{Digestion Coefficient} = \frac{100 - 100 \times \%\ \text{Indicator in feed} \times \text{Nutrient in Feces}}{\%\ \text{Indicator in Feces} \times \%\ \text{Nutrient in Feed}}$$

(Zewdie 2019). As horses digest their feed it is assumed the reference substance and the nutrient of the feed pass through the digestive tract at a uniform rate. Rate of excretion can vary with feed intake. The ratio of indicator and nutrients is consistent through a 24-hour period. A small amount of feces collected at any point in the day should be sufficient. However, collecting samples more than once a day will lead to a more accurate digestibility calculation. This method of sampling minimizes time, labor, and expense when compared to the total collection method. The main problem with this method of estimating digestibility is incomplete recovery of the indicator thus compromising the accuracy of the digestibility calculation.

In vitro digestion methods were first developed as an alternative to costly, labor intensive, time consuming, and ethically difficult *in vivo* methods for predicting digestibility (Tassone et. al., 2020). The first method for determining digestibility was the Tilley and Terry technique (TT) using a two-stage rumen fluid-pepsin process for cattle. Researchers have found while this technique worked well for fresh grasses, it did not favor silages or straw (Klopfenstein et. al., 1972). Goering and Van Soest modified the TT by replacing the acid-pepsin step with a neutral detergent step (Goering and Van Soest, 1970). This modification proved to be faster and more accurate, basing digestibility on the basis of undigested cell-wall constituents. Researchers found a clear linear relationship between the disappearance of NDF and the production of gas (Pell and Schofield, 1993). There was a need for a piece of apparatus capable providing

traditional *in vitro* digestibility analysis while eliminating some analytical errors pertaining to sample handling and manual filtration steps. This led to the development of the ANKOM Daisy II Incubator (ANKOM Technology, Macedon, NY, USA).

What initially started as a fragile wooden cabinet evolved into the more resistant metal cabinet used today. The ANKOM Daisy II Incubator is based off of *in vivo* simulation of digestion. The ANKOM Daisy II Incubator can hold up to 92 samples in a temperature-controlled chamber containing four glass jars. Samples are weighed into F57 filter bags (ANKOM Technology) and placed into the jars with inoculum and buffer solution. Each jar rotates inside the incubator and contain a perforated agitator baffle that separates samples while still allowing free movement of digestion medium. This perforated agitator baffle completely immerses bags at every spin of the jar. This prevents bags from floating to the top of the jar and accumulating gasses inside them. Bags are weighed before and after a set incubation period and the disappeared material is considered digestible dry matter. Recovery and filtration of the bags have been noted as sources of variability.

The F57 bag is made up of a polyethylene fiber with a three-dimensional filtration matrix that allows for the best substrate interaction with minimal particle loss (Tassone et. al., 2020). Grind size of the forage interacts with the pore size of the bag affecting the extent of feed disappearance. Procedures by the ANKOM Company suggest griding the forage sample to a size of 0.25g–0.5g for use in the filter bag with a 25μ pore size. This comes out to be a 10mg/cm^2 ratio of sample size to bag surface area (Vanzant et. al., 1998).

The inoculum used in the ANKOM Daisy II Incubator dictates how nutrients are digested and are also responsible for the greatest source of uncontrolled variation (Tassone et. al., 2020). The inoculum creates a similar environment as the digestive tract however, differences in

inoculum are noted between individuals. Rumen fluid was the first and most frequently used source of inoculum. The difficulties with collecting fresh rumen fluid are the cost, availability, and animal welfare issues associated with collecting it. Fresh rumen fluid requires access to a cannulated research animal, someone skilled with an esophageal tube, or collecting rumen fluid from freshly slaughtered cattle. Cannulation is a costly procedure and is often criticized on ethical grounds (Tassone et. al., 2020). After an animal is cannulated an abundance of maintenance is required. Maintenance includes long term care for the animal as well as a clean environment to avoid infections. Another way to collect rumen fluid, avoiding canulation, is to obtain samples via the esophagus. The downfall with this method is samples are not representative of the entire rumen and are often contaminated with saliva. The final option is to obtain rumen fluid at slaughter (Beyihayo et. al., 2015).

Due to the difficulty of collecting fresh rumen fluid, researchers started exploring the use of fresh feces as a viable form of inoculum. Limitations with the use of fecal material are present due to the anatomy of the bovine digestive system. Being cattle are foregut fermenters; the majority of fibrous plant material is broken down in the rumen. With the main site of digestion preceding the small intestine this allows for nutrients to be absorbed prior to defecation. Microbes escaping the rumen are often degraded in the abomasum and small intestine as protein sources. Only a small number of microbes survive the acidity of the abomasum and are present in the feces. Other limitations include lower enzymatic activity of fecal inoculate when compared to rumen fluid as well as variability of fecal inoculum preparation (Hughes et. al., 2012). However, studies have shown bovine fecal material can be a viable form of inoculum. A study by Laudadio found a significant relationship between the digestibility of forages using rumen

liquor and fecal liquor when comparing dry matter, organic matter, crude protein (CP), NDF, and ADF (Laudadio et. al., 2009).

Feces are frequently used as inoculum for *in vitro* incubation trials on monogastric animals. Access to cecally cannulated horses are often not readily available therefore researchers look to fresh fecal samples when studying the equine hindgut. Because horses are hindgut fermenters, microbial digestion is the last stage of digestion before defecation. Fecal microbial populations have a greater potential to be represented in the equine digestive system compared to the ruminant digestive system because the cecum succeeds the small intestine. Several studies have compared microbial diversity, structure, and activity in the hindgut and feces of the horse concluding the microbial communities found in the proximal region of the hindgut were clearly different from those found in the distal region (Da Veiga et. al., 2005; Hastie et. al., 2008; Dougal et. al., 2012 and 2013; Costa et. al., 2015; Grimm et. al., 2017). Researchers have reported volatile fatty acid and lactate concentrations were lower in the feces than the hindgut (Grimm et. al., 2017). Therefore, fecal material is representative of the microbial composition of distal hindgut but not the proximal region. Grimm reported cellulolytic, amylolytic and lactate-using bacteria were significantly correlated between the cecal and fecal samples (Grimm et. al., 2017). The core microbiome in the in the proximal large intestine (cecum, RVC, and RDC) include (in order of largest to smallest abundance) *Bacteroidales*, *Lachnospiraceae*, *Prevotellaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, and *Fibrobacteraceae*. The core microbiome in the distal large intestine (RDC, SC and feces) are dominated (in order of largest to smallest abundance) by *Precotellaceae*, *Fibrobacteraceae*, *Lachnospiraceae*, *Bacterodetes* and *Clostridiaceae*. The LDC is dominated by *Lachnospiraceae*, *Clostridiaceae 1*,

Bacteroidales, and *Erysipelotrichaceae* (Dougal et. al., 2013). Differences as well as overlap are apparent among the core microbiome in the hindgut of the horse.

Feces are identified as an inexpensive and readily available source of microorganisms easily collected from several animals within a short amount of time (Earing et. al.,2010). Lowman was the first to prove equine feces are a viable source of microbial inoculum and that fecal microflora can remain viable for several hours after excretion or collection (Lowman et. al., 1999). Lattimer indicated equine feces are an acceptable inoculum source for *in vitro* experiments using the ANKOM Daisy II Incubator (Lattimer et. al., 2007). A study by Earing compared *in vivo* methods to *in vitro* methods in horses (Earing et. al., 2010). *In vitro* incubation lengths of 30, 48, and 72 hours were used. Results indicated all *in vitro* DMD estimates for the alfalfa diet were less than the *in vivo* estimate. For the alfalfa oat, timothy, and timothy oat diets the 30- and 48-hour *in vitro* DMD estimates were less than *in vivo* estimates. However, the 72-hour incubation estimates for the previous three diets were not different than the *in vivo* estimates. Lack of change in the previous three diets was noticed between 48 and 72 hours indicating most DM digestion was complete by 48 hours. This suggests the alfalfa diet required a longer incubation period to reach DMD similar to *in vivo* results. Earing reported that although *in vitro* DMD estimates were consistently less than the *in vivo* estimates at 30 and 48 hours, the diets still ranked in the same order (Earing et. al., 2010). Researchers concluded the 72-hour incubation period resulted in the highest similarity between the *in vivo* method and *in vitro* method.

Fecal Inoculum Storage

In vitro digestibility trials are typically performed using fresh microbial inoculum. The ability to store feces would be immensely beneficial for researchers with limited access to fresh

feces or laboratory assistance. The storage of equine feces allows samples to be collected further distances from the research facility or to be processed at a later date. However, limited information is known about how storage temperature and storage time affect microbes within the sample.

Relevant studies on how storage temperature affect microbiota in veterinary species is limited. Equine fecal microbiota also varies significantly per horse and location (surface versus center) of fecal samples (Stewart et. al., 2018; Beckers et. al., 2017). A study by Stewart compared fecal microbiota collected directly from the rectum versus the stall floor, the center versus the surface of the fecal ball, and the duration of environment exposure (Stewart et. al., 2018). Individual bacterial taxa were significantly different with both sample location and collection time, but remained fairly stable up to 6 hours for center fecal samples (Stewart et. al., 2018). Beckers collected samples at 0, 2, 4, 6, and 12 hours after defecation and then froze them at -20˚C after collection. Microbial DNA was extracted using the MoBio PowerSoil DNA Isolation kit (Beckers et. al., 2017). Researchers found a significant decrease in diversity at the 12-hour time point. A study by Bustamante also concluded samples exposed to aerobic conditions for more than 12 hours showed a significant decrease in microbial composition (Bustamante et. al., 2021). One study froze fecal inoculum at -20˚C for 24, 48, and 72 hours and reported an effect on extent and rate of *in vitro* substrate fermentation with reported differences being dependent upon the nature of the substrate used (Murray et. al., 2012). Samples collected 24 hours after defecation are acceptable for use however, researchers must expect bacterial populations to deviate.

O'Donnell researched how storage conditions including 39˚C for 15 minutes, 22˚C for 6 hours, 3˚C for 6 hours and -18˚C for 24 hours altered the viability of microbial inoculum used for

in vitro equine digestibility trials (O'Donnell et. al., 2021). The study concluded fecal material held at 39˚C immediately used and fecal material held at 22˚C for 6 hours yielded similar DMD, NDF, and ADF results. Concluding short term storage of fecal material for microbial inoculum is possible (O'Donnell et. al., 2021). Fecal samples stored at 3˚C for 6 hours and -18˚C for 24 hours adversely reduced digestibility values. Signifying that cold storage of fecal material is not a viable option for forming microbial inoculum for digestibility studies.

Fecal storage was used in a study by Kopper for microbiota transplant use in veterinary patients (Kopper et. al., 2021). Researchers stored manure at -20˚C for up to 4 weeks to be passed through a simulated proximal gastrointestinal tract. Results indicated storage at -20˚C for greater than 1 week significantly decreased viability of the microbial population. Most significantly impacting gram-negative enteric bacteria (Kooper et. al., 2020).

METHODS AND MATERIALS

Three mature quarter horse geldings were selected from the herd of horses at Missouri State University Darr College of Agriculture. Horses ranged in weight from 450–550 kilograms and in age from 15 ± 6 years of age. All animals maintained a body condition score of five during the entirety of the study. All procedures involving the care, management, and use of horses for the study were approved by the Institutional Animal Care and Use Committee of Missouri State University (see Appendix). All animals were housed in covered 3.6x7.3 meter pens with limestone footing. Animals were fed the same ration twice a day at 0800 and 1700h. Rations consisted of 3.63 kg of concentrate (Easy Keeper Edge, MFA Inc., Columbia, MO) and 10.88 kg of a mixed grass fescue hay with ad libitum access to clean water. Nutritional values for concentrate and fescue hay are noted in Table 1. The three geldings used for this study had been maintained on this ration of concentrate and mixed grass fescue hay prior to the study. Equines were given ten days to adjust to any change caused by receiving a precisely weighed diet.

Table 1. Chemical composition (%) of concentrate (Easy Keeper Edge, MFA) and fescue hay Diet Provided

Nutrient	%DM	%ADF	%NDF	%CP
Concentrate	90.67	22.99	40.03	15.44
Fescue Hay	92.29	39.48	59.32	9.28

DM, matter; ADF, acid detergent fiber; NDF neutral detergent fiber; CP, crude protein

Fecal Samples

Fecal samples were obtained from the same three geldings on three separate days over a two-week period prior to feeding at 0800 per rectum using lubrication and disposable rectal sleeves. Fecal samples were sealed in individual airtight bags. Although bags were not maintained under fully anerobic conditions, care was taken to expel as much air as possible before sealing. Sealed samples were then placed in a cooler filled with warm water at 39˚ C to maintain a consistent temperature. Samples remained in the cooler for 5 ± 2 minutes while being transported to the laboratory. Once at the laboratory 15 g of each fecal sample was pooled to make a single 45 g sample. A total of four aliquots were formulated, stored in an open Ziplock® baggie exposed to aerobic conditions, and placed in their respective storage conditions.

Four storage conditions were evaluated in this study. Two pooled samples (A and B) were placed on the counter of the climate-controlled laboratory in a 23 ± 1˚C environment. The other two pooled samples (C and D) were placed in a refrigerator set at 12 ± 1˚C. Samples A and C were processed 6 hours after being collected while samples B and D were processed 12 hours after being collected. For this study the control was selected as sample A held at 23˚C for 6 hours. This treatment was chosen based on research from O'Donnell (O'Donnell et. al., 2021).

Forage Samples

Prior to the first sampling period, nine forage samples were collected. Representative samples were taken from a variety of forages including mixed grass hay, and alfalfa hay ranging in age from one to three years. Samples were collected by coring individual bales and randomly selecting from a bag of alfalfa cubes. Forage samples were dried in a 50 ± 2˚C oven for 48 hours then ground using a Cyclone Sample Mill equipped with a 1.0 mm screen. Forage samples were

sent to a professional laboratory (Custom Laboratory, Monett, MO) for wet chemistry analysis of dry matter, NDF, ADF, and CP presented in Table 2.

Samples were stored in sealed plastic bags inside a desiccator containing drietrite until further use. F57 filter bags were prepared for use in the ANKOM Daisy II Incubator. Filter bags were soaked in acetone for five minutes and laid out on a wire rack to dry for an additional five minutes. Bags were labeled according to forage sample, jar specification, and treatment. After being labeled, the weight of each empty F57 filter bag was recorded. Samples were weighed with a sartorius milligram balance with an accuracy of ±.0001. The scale was zeroed and approximately .50 g of forage sample was added. Forage bags, now full of sample, were sealed with a 120V Impulse Heat Sealer (American International Electric, South El Monte, California). Each jar contained duplicates of every sample as well as a blank bag without a forage sample. This blank bag allows adjustment for loose forage particles that have attached themselves to the bag while in the digestion jars. Each digestion jar contained a total of 19 filter bags.

In Vitro Digestibility

For the study two separate buffer solutions were created and combined for use in the ANKOM Daisy II Incubator. Buffer solution A was created by combining KH_2PO_4 10 g/liter; $MGSO_4 * 7\ H_2O$ 0.5 g/liter; NaCl 0.5 g/liter; $NaCl_2 * 2H_2O$ 0.1 g/liter; Urea (reagent grade) 0.5 g/liter. Buffer solution B was created by combining Na_2CO_3 15.0 g/liter with $Na_2 * 9\ H_2O$ 1.0 g/liter. The final buffer solution was made by combining 1330 ml of solution A with 266 ml of solution B (1:5 ratio) to obtain a final pH of 6.8. After combining, mixed buffer solution was heated to 39˚C using a Lab Companion stirring hot plate (Lab Companion, Billerica, Massachusetts) and 1600 ml of the A/B mixture was added to each digestion jar. Jars were then

placed in the ANKOM Daisy II Incubator and allowed to equilibrate for a minimum of thirty minutes.

Inoculum for the digestion jars was formed by placing the 45 g fecal sample in a blender with 400ml of distilled water at 39˚C . Carbon dioxide gas was dispensed over the top of the sample for 15 seconds before closing the lid and blending with a standard kitchen blender for 15 seconds. The inoculum was then strained through four layers of cheese cloth. Contents were squeezed by hand to extract as much inoculum as possible. Inoculum solution and prepared F57 filter bags were then added to each jar. Jars were purged with CO_2 gas for 30 seconds before securing the lid.

Sample Analysis

Samples were allowed to incubate in the ANKOM Daisy II Incubator for 48 hours at a maintained temperature of 39.5˚C ± 0.05. Samples B and D were added to the ANKOM Daisy II Incubator 6 hours after samples A and C. Addition and removal of jars was done quickly and temperature change was negligible. At the completion of incubation, fluid was drained, and sample bags were rinsed with cold tap water until water ran clear. Filter bags were placed in a drying oven at 50 ˚C ± 1 and left for a minimum of 24 hours. Dried samples were weighed to obtain the final bag weight after *in vitro* treatment. Percentage *in vitro* true digestibility was determined as [(100-(final bag weight after treatment-(initial bag weight x correction Factor))) ÷ (initial sample weight x dry matter)] x 100 = %IVTD $_{DM}$.

Statistical Analysis

Data from the 2×2 factorial design with nine forages and three replicates conducted over a two-week period were analyzed by ordinary least squares (OLS) regression. These forages had varying levels of NDF, ADF, and CP. Storage conditions affects were estimated as the difference between the storage condition of 12˚C for 6 hours and the remaining experimental storage conditions. Alfalfa cubes were chosen as the baseline forage. Dummy variables were included in the model to account for differences between forage type.

Seven models were used in the OLS regression. Models 1 through 3 only compare treatment time by treatment temperature, Model 4 takes into account forage affects while model 5 takes into account replicate affects. Model 6 does not include forage by replicate interactions while model 7 does.

Table 2. Forage chemical composition and nutrient analysis of dry matter, neutral detergent fiber, acid detergent fiber and crude protein (DM basis).

	Chemical Composition of Forage			
Forage Sample	%DM	%NDF	%ADF	%CP
Alfalfa Cubes	92.55	48.01	37.64	15.67
Alfalfa Hay 1	92.33	47.02	34.85	19.05
Alfalfa Hay 2	91.82	40.65	32.56	16.92
Mixed Grass Hay 1	92.92	56.03	39.66	9.67
Mixed Grass Hay 2	92.82	67.50	45.41	9.33
Mixed Grass Hay 3	92.29	59.32	39.48	9.28
Mixed Grass Hay 4	92.85	61.08	41.80	7.34
Mixed Grass Hay 5	92.87	59.99	39.92	7.28
Mixed Grass Hay 6	92.91	70.56	49.8	7.12

Chemical composition of forage parameters measured using a 50 ± 2˚C oven for dry matter (DM), wet chemistry for neutral detergent fiber (NDF) and acid detergent fiber (ADF), and NIR spectrometry for crude protein (CP).

RESULTS

Dry matter digestibility for each time, temperature, and forage type are displayed by trial in tables four through seven. Averages are displayed for each trial and mean of collective trials for each forage in their respective time and temperature. Table 3 reflects the DMD for all nine forages at 23˚C for 6 hours. Table 4 reflects the DMD for all nine forages at 12˚C for 6 hours. Table 5 reflects the DMD for all nine forages at 23˚C for 12 hours. Table 6 reflects the DMD for all nine forages at 12˚C for 12 hours. All forages are ordered based on CP values reported in Table 2.

The mean values from tables four through seven are combined in Figure 1. Digestibility of forages differs based on forage type, but no differences were seen due to treatment affect. Standard error bars were included in Figure 1. To improve the quality of the figure. Forages with lower NDF and ADF (AC, AH1, AH2) had higher DMD than those with higher NDF and ADF (MGH 2, MGH 4, MGH 6). Finally, OLS analysis indicated there were no treatment affects within forage (Table 3).

Table 2 displays the DM, ADF, NDF, and CP for each forage used in the study. Forages were ordered based on CP values. The OLS regression in Table 7 compared environmental conditions and DMD of forages to a baseline temperature of 12˚C, time of 6 hours and forage: alfalfa cubes. Model 1 compared 12 hours at 12˚C hours without forage affect to the baseline temperature of 6 hours and 12˚C and found no significant difference (P>0.05). Model 2 compared 6 hours at 23˚C without forage affect to baseline temperature and time resulting in no significant difference (P>0.05). Model 3 compared 12 hours at 23˚C without forage affect to the baseline temperature and found no forage by temperature or forage by time interaction. Model 4

compared all storage conditions to the baseline time and temperature as well as compared forage affect to the baseline forage alfalfa cubes. This model reported no significant difference (P<0.05) in storage conditions. Here there was a significant forage affect for AH 1, AH 2, MGH 2, MGH 4, MGH 5, MGH 6 (P<0.05). Model 5 compared all environmental conditions to baseline conditions and compared variation between replicates. A significant difference (P<0.05) among all replicates was recorded. Model 6 compared all environmental conditions, forage affect, and replicates to the baseline temperature, time, and forage. A significant difference (P<0.05) was reported between AH 1, AH 2, MGH 2, MGH 4, MGH 5, and MGH 6, as well as a significant difference (P<0.05) between replicates. Model 7 compared all storage conditions, forage affect, and variation between replicates as well as interactions. A significant difference (P<0.05) was reported between AH 2, MGH 2, MGH 3, MGH 4, MGH 5, MGH 6 as well as between replicates two and three. Regardless of replicate effect, forage effect, or interactions, the difference between storage condition is not significant (P>0.05).

Table 3. Average percent (± S.D.) dry matter digestibility (%DMD) by forage and repetition when fecal samples were stored for 6-hours at 23˚C under aerobic conditions.

Forage	Repetition			
	Trial 1	Trial 2	Trial 3	Mean
Alfalfa Cubes	40.25±0.59	49.13±4.37	46.46±1.48	45.28±4.58
Alfalfa Hay 1	39.87±0.66	54.36±0.50	49.60±2.19	47.94±6.69
Alfalfa Hay 2	50.33±4.25	58.72±3.30	57.63±0.93	55.56±4.76
Mixed Grass Hay 1	29.08±1.55	33.85±1.76	36.00±2.01	32.98±3.46
Mixed Grass Hay 2	18.45±2.98	25.05±0.03	25.04±1.96	22.85±3.76
Mixed Grass Hay 3	29.70±1.50	32.61±2.25	35.45±1.28	32.59±2.90
Mixed Grass Hay 4	17.55±1.40	22.52±2.29	24.14±0.11	21.40±3.30
Mixed Grass Hay 5	22.26±0.30	26.18±1.10	28.58±0.38	25.67±2.90
Mixed Grass Hay 6	13.13±0.75	18.96±1.72	22.89±0.44	18.33±4.48

Table 4. Average percent (± S.D.) dry matter digestibility (%DMD) by forage and repetition when fecal samples were stored for 6-hours at 12˚C under aerobic conditions.

Forage	Repetition			
	Trial 1	Trial 2	Trial 3	Mean
Alfalfa Cubes	41.88±2.35	48.41±0.36	46.46±1.48	45.88±3.32
Alfalfa Hay 1	42.06±0.31	53.78±0.79	48.53±0.25	48.13±5.27
Alfalfa Hay 2	52.08±1.16	58.50±1.50	57.26±0.26	55.95±3.16
Mixed Grass Hay 1	28.59±3.61	32.95±0.48	35.09±3.49	32.21±3.72
Mixed Grass Hay 2	20.91±2.98	26.24±6.05	23.15±0.34	23.43±3.85
Mixed Grass Hay 3	30.67±1.87	32.43±0.71	33.89±0.37	32.33±1.70
Mixed Grass Hay 4	19.77±0.84	23.38±2.90	19.39±01.84	20.85±2.52
Mixed Grass Hay 5	22.89±2.62	28.85±0.54	30.59±0.67	27.45±3.82
Mixed Grass Hay 6	17.45±2.64	19.52±0.05	21.63±1.97	19.53±3.72

Table 5. Average percent (± S.D.) dry matter digestibility (%DMD) by forage and repetition when fecal samples were stored for 12-hours at 23˚C under aerobic conditions.

Forage	Repetition			
	Trial 1	Trial 2	Trial 3	Mean
Alfalfa Cubes	43.97±1.43	47.76±1.25	49.20±1.60	46.98±2.66
Alfalfa Hay 1	40.50±0.55	52.34±0.30	51.93±2.57	48.26±6.13
Alfalfa Hay 2	50.40±1.45	60.46±0.48	60.48±0.01	57.11±5.25
Mixed Grass Hay 1	32.12±0.12	35.45±0.41	34.23±0.69	33.93±1.55
Mixed Grass Hay 2	20.81±4.25	25.53±2.78	20.85±1.01	22.40±3.36
Mixed Grass Hay 3	31.44±1.12	37.24±0.69	35.13±0.95	34.60±2.72
Mixed Grass Hay 4	17.18±0.71	25.52±5.48	23.95±0.34	22.22±4.67
Mixed Grass Hay 5	26.80±3.66	34.59±9.35	26.56 ±0.53	29.33±6.07
Mixed Grass Hay 6	12.55±1.60	24.59±1.27	18.83±.89	18.66±5.48

Table 6. Average percent (± S.D.) dry matter digestibility (%DMD) by forage and repetition when fecal samples were stored for 12-hours at 12˚C under aerobic conditions.

Forage	Repetition			
	Trial 1	Trial 2	Trial 3	Mean
Alfalfa Cubes	42.44±1.94	50.46±1.52	46.19±0.23	46.36±3.76
Alfalfa Hay 1	36.18±21.00	52.19±0.49	48.59±3.37	45.65±12.15
Alfalfa Hay 2	54.62±2.27	58.99±2.13	57.78±2.18	57.13±2.64
Mixed Grass Hay 1	30.66±0.81	35.03±2.84	37.34±2.75	34.34±3.53
Mixed Grass Hay 2	22.49±2.81	24.48±0.72	24.14±0.85	23.70±1.66
Mixed Grass Hay 3	28.67±2.01	35.41±0.56	35.48±2.12	33.19±3.74
Mixed Grass Hay 4	20.89±0.94	24.91±0.61	26.89±0.74	24.23±2.80
Mixed Grass Hay 5	24.90±0.84	30.21±0.31	30.45±1.16	28.52±2.88
Mixed Grass Hay 6	16.39±2.18	21.59±0.32	22.80±2.43	20.26±3.38

Table 7. Ordinary least squares (OLS) regression analysis of dry matter digestibility percentages based on storage condition, forage, and replicate interactions

Interactions	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7†
12h × 12˚C	1.03	1.03	0.85	0.85	0.85	0.85	0.85
	(1.76)	(1.77)	(2.50)	(1.29)	(2.45)	(1.24)	(1.19)
6 h × 23˚C		−0.17	−0.35	−0.35	−0.35	−0.35	−0.35
		(1.77)	(2.50)	(1.29)	(2.45)	(1.24)	(1.19)
12h × 23˚C			0.36	0.36	0.36	0.36	0.36
			(3.54)	(1.83)	(3.47)	(1.76)	(1.68)
AH 1				11.28***		11.82***	−2.48
				(1.73)		(1.67)	(3.09)
AH 2				20.22***		20.76***	9.72***
				(1.73)		(1.67)	(3.09)
MGH 1				−2.85		−2.31	−12.02***
				(1.73)		(1.67)	(3.09)
MGH 2				−13.12***		−12.58***	−21.47***
				(1.73)		(1.67)	(3.09)
MGH 3				−3.04*		−2.50	−12.01***
				(1.73)		(1.67)	(3.09)
MGH 4				−14.04***		−13.50***	−23.29***
				(1.73)		(1.67)	(3.09)
MGH 5				−8.47***		−7.93***	−17.92***
				(1.73)		(1.67)	(3.09)
MGH 6				−21.34***		−18.10***	−27.25***
				(2.60)		(2.62)	(3.09)
Replicate Two					6.45***	4.44***	−7.08***
					(2.12)	(1.12)	(2.67)
Replicate Three					5.66***	3.64***	−7.72***
					(2.12)	(1.12)	(2.67)
Constant	33.80***	33.88***	33.97***	35.88***	29.94***	32.64***	41.79***
	(1.25)	(1.53)	(1.77)	(1.32)	(2.12)	(1.50)	(2.30)
R2	0.00	0 00	0 00	0.74	0.05	0.77	0.80
Adj. R^2	−0.00	−0.01	−0.01	0.73	0.03	0.75	0.77
N	216	216	216	216	216	216	216

OLS estimates with standard errors in parentheses.
***$p < 0.01$; **$p < 0.05$; *$p < 0.1$, † model includes Forage × Replicate interactions.
Baseline temperature is 12◦C, baseline forage is Alfalfa Cubes, baseline time and 6 Hours.

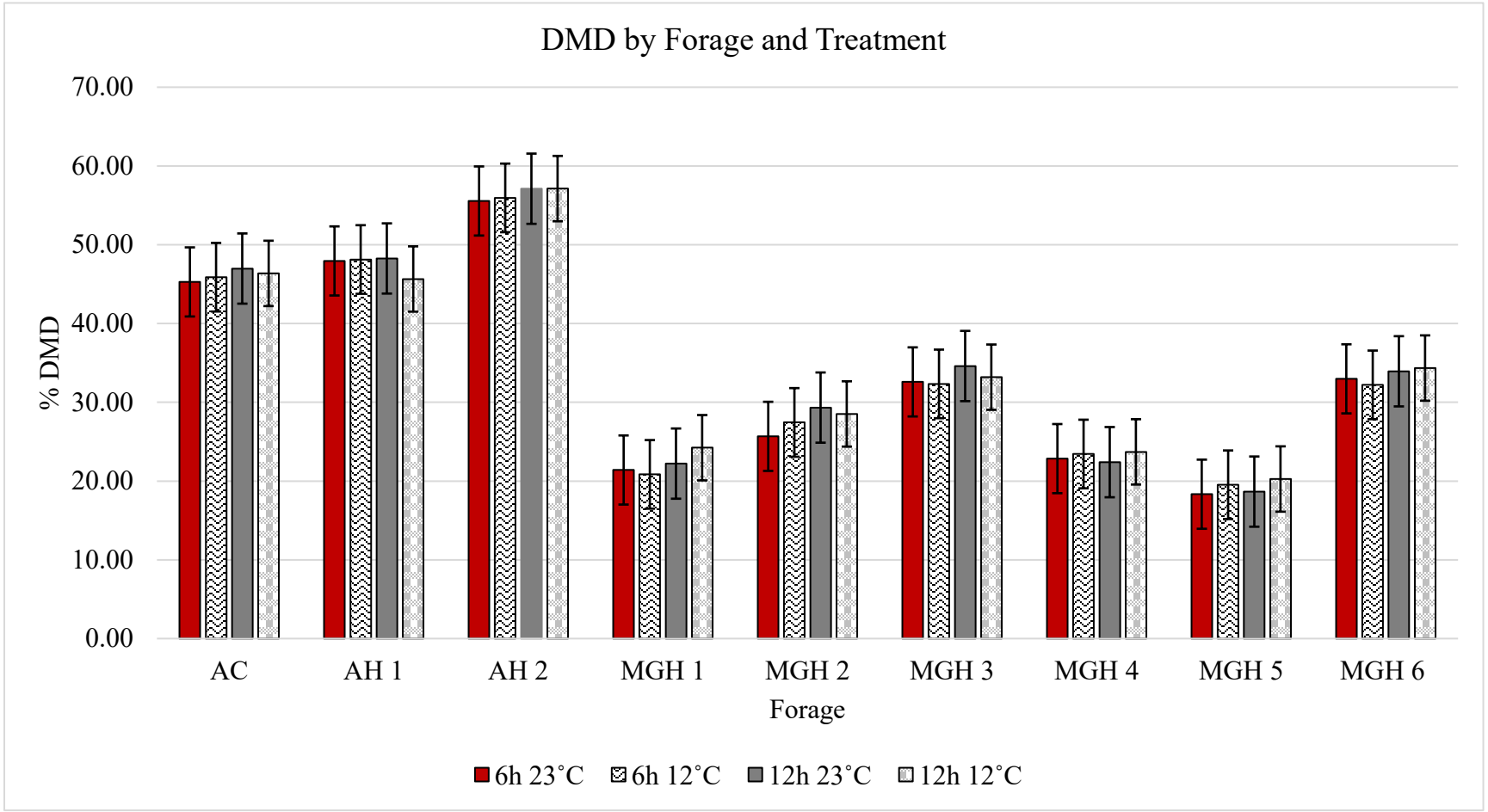

Figure 1. Equine dry matter digestibility of hay by microbial inoculum storage temperature, grouped by forage sample. AC=Alfalfa Cubes; AH1 = Alfalfa Hay 1; AH2 = Alfalfa Hay 2; MGH 1 = Mixed Grass Hay 1; MGH 2 = Mixed Grass Hay 2; MGH 3 = Mixed Grass Hay 3; MGH 4 = Mixed Grass Hay 4; MGH 5 = Mixed Grass Hay 5; MGH 6 = Mixed Grass Hay; Standard error bars included to improve the quality of the figure.

DISCUSSION

In this study no difference in digestibility was seen between microbial inoculum formed from a 6-hour sample versus a 12-hour sample exposed to aerobic conditions at 23˚C or 12˚C. Analysis of DMD demonstrated fecal material held under four different storage conditions provided microbial inoculum that, when incubated with forage samples, yielded similar digestibility results amongst samples tested. These results indicate short term storage or collecting natural deposited fecal material subject to 12˚C–23˚C temperatures for 6–12 hours exposed to aerobic storage conditions may be a viable source of microbial inoculum.

The statistical analysis presented in Table 3 indicates a significant (P<0.05) effect among forage types when compared to alfalfa cubes. This result was expected as forage NDF and ADF values ranged from 40.65 to 0.56 and from 32.56 to 49.8 respectively, were specifically chosen to highlight any forage treatment interactions. A visual comparison of DMD by forage and treatment can be made in Figure 1. An apparent difference is visible among the height of each cluster of bars representative of each forage sample. Difference among DMD is consistent with assorted levels of NDF and ADF. Forages with lower NDF and ADF values were more digestible by microbial inoculum while forages with high NDF and ADF values were less digestible. Digestibility was not reliant on fecal inoculum but rather forage quality. Overlapping standard error bars indicate no significant difference (P>0.05) within each cluster by forage and that storage condition of fecal inoculum did not change DMD for forage regardless of forage quality.

While it is known using fresh fecal samples is a viable source of microbial inoculum for *in vitro* studies (Lowman et. al., 1999; Latimer et. al., 2007; Earing et. al., 2010) storage conditions for this study were chosen based on results by O'Donnell (O'Donnell et. al., 2021).

Previous research concluded storage at refrigerator and freezer temperatures, 3˚C and -18˚C respectively, reduced estimated digestibility values, indicating cold storge is not a viable option for fecal material used to form microbial inoculum for forage digestibility studies. Researchers discovered material processed immediately at 39˚C and after 6 hours at 22˚C provided microbial inoculum that yielded similar digestibility results. The current study chose to use storage conditions consistent with O'Donnell's 6 hour sample at 22˚C, along with a temperature slightly warmer than the 3˚C household refrigerator, and a time slightly longer than the 6 hour time frame (O'Donnell et. al., 2021). Thus, storage conditions of 23˚C and 12˚C were chosen along with 6 and 12 hour time frames. The 12˚C temperature was specifically selected due to readily available access to a refrigerator being maintained at this specific temperature. Environmental conditions ranging between 23˚C and 12˚C are likely to occur outside of the laboratory. Therefore, this provides a realistic temperature fecal samples may be exposed to when collecting naturally deposited samples.

No significant difference ($P>0.05$) was seen between storage conditions. Referring to models 6 and 7 in Table 7, a significant ($P<0.05$) forage effect did occur, however no significant difference ($P>0.05$) impacted by time or temperature were recorded. Regardless of replicate effect, forage effect, or variation the difference between storage conditions was not significant ($P>0.05$).

Previous research has established the majority of the microbiome in the hindgut of the horse are oxygen sensitive, anaerobic organisms, and it is expected bacteria would not survive when placed in an aerobic environment (Julliand and Grimm, 2016). Consistent with published research, in the current study, fecal samples exposed to aerobic conditions remained fairly stable for up to 6 hours (Stewart et. al., 2018; O'Donnell et. al., 2021). However, inconsistent with

some studies, a significant decrease in microbial composition at 12-hours was not seen based on DMD results (Beckers et. al., 2017; Bustamante et. al., 2021).

The inability to notice a significant shift in microbial population is most likely due to the fact that the current study did not have the resources to sample and record individual microbes present in fecal samples and compare microbial population from the time of collection to the time of incubation under the four storage conditions. The present study only measured microbe performance on the basis of fecal inoculum interaction with forage samples and the resulting DMD. Incorporating a storage condition of feces at 39˚C processed immediately as the control would have given a better understanding of the microbial population present before aerobic conditions and the effect of storage conditions on DMD. Measuring inoculum pH with a pH meter before and after incubation would also help to gain a better understanding of microbe viability.

The restriction with adding a fresh fecal sample is the number of jars present in the ANKOM Daisy II Incubator. With 4 jars and 5 samples, the addition of processing fresh forage would result in the elimination of a storage condition, running 20 total trials to eliminate variation for the odd number of storage conditions, or the purchase of another ANKOM Daisy II Incubator. Eliminating a storage condition would interrupt the current 2 × 2 model used in the current study. The option of eliminating one was not appealing as then researchers would not be able to accurately determine if storage time or storage temperature were an independently factor contributing to any differences seen. The option of adding a storage condition was unappealing as it would require more labor and resources which were not available. The final option of purchasing an additional ANKOM Daisy II Incubator was also impractical as the funds were not

available. With the addition of a second incubator, variation could occur between the independent cabinets.

This study used microbial inoculum pooled from fecal samples of three quarter horse geldings. Previous studies have confirmed substantial variation in microbial communities among individuals (Carroll et. al., 2012; Stewart et. al., 2018; Beckers et. al., 2017). Pooling samples in the current study allowed for the elimination of individual variability as a source of error in the results. However, it is not known if pooled fecal samples provide microbial inoculum that present true average of digestibility results. Research by Stewart concluded bacterial taxa were significantly different when collected from the exterior of the fecal ball (exposed to aerobic conditions) vs. the interior of the fecal ball (somewhat protected from aerobic conditions)(Stewart et. al., 2018). In the current study fecal samples were broken apart to be weighed, and then mixed by hand before being placed in one of the four storage conditions. This weighing and mixing disturbed the structure of individual fecal balls and exposed fecal samples to aerobic conditions inconsistent with naturally deposited fecal material. This mixing had the potential to overexpose microbes to aerobic condition potentially damaging a greater number of microbes than anticipated. This would only be known if DNA analysis of present microbes was taken at the time of incubation.

Access to a cecally canulated horse could also provide an interesting comparison to the current study. The progression of the microflora from the cecum to the feces, and how they are impacted based on storage conditions would lead to a very robust study. This insight would allow researchers to clearly compare how the ideal inoculum source compares to an expired fecal sample.

The limited sample size used for this study allow for a decrease in accuracy due to experimental error. The use of a larger number of feal samples as well as more storage conditions used could lead to a more accurate study. Measuring DNA analysis of microbes present in each individual fecal sample would create a better understanding of how aerobic conditions impact fecal samples at the storage conditions used for this study.

CONCLUSION

Results indicate fecal material collected at 12-23˚C and between 6-12 hours is a viable source of fecal inoculum for *in vitro* digestibility studies when immediate collection or processing of fecal samples is not available. Verifying stored fecal samples provide adequate microbial inoculum under varying conditions allows researchers to use naturally deposited fecal material, collect samples further distances from the laboratory, and aid in standardizing *in vitro* research procedures and results. Samples at 23˚C for 6-hours, 12˚C for 6 hours, 23˚C for 12 hours and 12˚C for 12 hours all resulted in similar DMD indicating there was no significant difference among samples. Further research will need to be conducted to determine if exposing naturally deposited fecal material to longer lengths of aerobic conditions, humidity, and environmental contamination alter the viability of fecal inoculum.

Dissimilar to Bstameante fecal samples in the current study remained viable up to 12 hours post collection (Bstameante et. al., 2021). This result could be due to the mixing of different samples. Although some microbes could have died due to aerobic conditions the strongest microbes of each sample could be providing an overestimation of true digestibility. Keeping fecal samples separated would provide more accurate information on how aerobic conditions affected the microbes of each sample. The ability to collect microbial DNA would also be beneficial to understanding the microbes strong enough to survive aerobic conditions and microbes that are not. An understanding of microbe resilience would provide more accurate knowledge of DMD that occurs.

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APPENDIX: MISSOURI STATE IACUC APPROVAL

July 30, 2020

RE: IACUC protocol 2020-10

Hello Gary,

Your IACUC protocol #2020-10 entitled "Effect of Storage and Temperature on Use of Equine Fecal Samples as a Source of Microbes for In-Vitro Digestion of Forage" has been approved.

The final approval date is 07/27/2020.

Thank you and if you need anything in the future regarding this protocol please contact me either via email (IACUC@missouristate.edu) or at 417-836-8419.

Sincerely,

Janene Proctor