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# Impact of Storage Temperature on Viability of Microbial Inoculum for Estimating In Vitro Equine Digestibility

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# IMPACT OF STORAGE TEMPERATURE ON VIABILITY OF MICROBIAL INOCULUM FOR ESTIMATING IN VITRO EQUINE DIGESTIBILITY

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By

Delaney Brenne O'Donnell

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**IMPACT OF STORAGE TEMPERATURE ON VIABILITY OF MICROBIAL INOCULUM FOR ESTIMATING IN VITRO EQUINE DIGESTIBILITY**

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## ABSTRACT

This study evaluated the effect of storage temperature of equine fecal material on the viability of microbial inoculum used for in vitro equine digestibility trials. Fecal material was stored at four storage temperatures of 39°C for 15 minutes (control), 22°C for 6h, 3°C for 6h, and -18°C for 24h. Stored fecal material was used to form microbial inoculum for use in an Ankom Daisy II Incubator. Six 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. After determination of dry matter digestibility (DMD), an Ankom$^{2000}$ Fiber Analyzer was used to measure neutral detergent fiber digestibility (NDFD) and acid detergent fiber digestibility (ADFD). A mixed linear model was used to analyze differences in DMD, NDFD, and ADFD. Further analysis with a mixed linear model was used to evaluate the effect of forage quality parameters crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) as covariates for DMD, NDFD, and ADFD results. Significant differences were observed for DMD ($P<0.0001$), NDFD ($P<0.01$), and ADFD ($P<0.001$) between 39°C and 3°C, 39°C and -18°C, 22°C and 3°C, and 22°C and -18°C. No differences ($P>0.05$) were observed in DMD, NDFD, or ADFD between 39°C and 22°C or 3°C and -18°C. Covariance analysis found no difference ($P>0.05$) in slope between any of the four treatment levels when CP, NDF, and ADF were modeled as covariates for DMD, NDFD, and ADFD. Results show that fecal material stored for up to six hours in 22°C temperature conditions provides a viable alternative to fresh fecal material for the formation of microbial inoculum. Chilled and frozen fecal material did not provide comparable microbial inoculum to the control. Further research is needed to determine the reason for the decline in DMD, NDFD, and ADFD observed when microbial inoculum was formed using chilled and frozen fecal samples.

**KEYWORDS**: equine, digestibility, microbial inoculum, in vitro, inoculum storage, microbial population

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# INTRODUCTION

## Need for Study

Microbial populations in the equine hindgut are highly variable and shift depending on the composition of the diet, with a consistent but small core population of bacteria identified across all individuals (Ericsson et al., 2016). Performance animals are often supplemented with high-fat or high-starch feeds in order to prevent the caloric deficit that would be present on a forage-only diet. High-fat and high-starch diets have been shown to cause shifts in the microbial population that reduce the core bacterial community in the hindgut, providing one avenue for explaining the higher prevalence of diseases caused by gastric disturbances and microbial disruption in this population of equines (Dougal et al., 2014). Research evaluating the digestibility of equine diets provides avenues for developing diets that better meet the nutritional requirements of equines at all lifestages. Studies evaluating differences in digestibility caused by shifts in hindgut microbial populations provide for more accurate diet formulation and may help explain the increased incidence of gastric disturbances in horses on high-starch diets.

In vitro digestibility studies provide a faster, more accessible, and more cost-effective way of evaluating the digestibility of equine diets than traditional methods using in vivo techniques while also providing opportunities for the evaluation of diets that cannot be studied via in vivo digestibility trials, such as diets based on low-quality forages that would not meet maintenance nutrient requirements. Additionally, in vitro batch culture techniques allow for the simultaneous study of multiple feedstuffs and are less labor intensive than in vivo digestibility trials (Lowman et al., 1999; Lattimer et al., 2007; Earing et al., 2010).

Use of equine feces as the source of microbial inoculum for in vitro digestibility research using the Daisy II Incubator has been previously validated (Lattimer et al., 2007; Earing et al., 2010). All previous studies have used freshly collected fecal material as the source of microbial inoculum. There is a lack of research on the effects of temperature and environment on the fermentative ability of microbes present in equine feces. Results from Murray et al. (2012) suggests that the effect of storage on the fermentative capacity of fecal material is dependent on the quality of forage being digested. Research on the use of preserved inoculum sources would provide researchers with the ability to use inoculum from animals located further distances from laboratory facilities, to transport inoculum in a variety of temperatures and environmental conditions, and would aid in standardizing in vitro research procedures and results (Murray et al., 2012).

**Objectives**

The purpose of this study was to evaluate the effect of storage conditions of microbial inoculum on dry matter digestibility, neutral detergent fiber digestibility, and acid detergent fiber digestibility. A further objective was to determine if there is an interaction between forage chemical composition and the effect of storage conditions of microbial inoculum on dry matter digestibility, neutral detergent fiber digestibility, and acid detergent fiber digestibility.

**Null Hypothesis**

The null hypothesis of this study was that storage conditions of the fecal material used to form microbial inoculum does not affect measured estimates of dry matter digestibility, neutral detergent fiber digestibility, or acid detergent fiber digestibility. Further, that there was not an

interaction between storage conditions of microbial inoculum and the chemical composition of the forage samples digested in the in vitro system.

# LITERATURE REVIEW

## Equine Digestion

Equines are part of a group of herbivores that have evolved to be reliant on fiber as a primary energy source. The gastrointestinal tract (GIT) reaches a length of 30m and a volume of 150L in the adult animal. Proteins, soluble carbohydrates, and fats are all primarily digested and absorbed in the small intestine (SI). A vast symbiotic microbial community in the large intestine (LI) ferments structural components of ingested fiber into short-chain fatty-acids that can be utilized by the animal for energy (Ericsson et al., 2016). Due to increased nutritional requirements from athletic demands placed on equines used for recreation and competition, many animals are supplemented with high energy feedstuffs, either in the form of high-starch feeds like grains, or high-fat feeds, like oils (Dougal et al., 2014).

Mastication in the mouth starts the process of digestion. Chewing and grinding actions reduce the particle size of feed, increasing the surface area available for enzymes and microbes to act on later in the GIT (Geor et al., 2013). After mastication of a forage meal the average particle size of forage residues is 0.74-1.17mm (Clauss et al., 2014). Saliva produced in the mouth serves to lubricate boluses of feed to ease passage through the esophagus and provides bicarbonate to act as a buffer within the stomach. Transit through the 1.3m long esophagus is rapid, at 4-10 seconds per swallowed bolus of feed (Geor et al., 2013).

Feed spends 0.5-3hr in the stomach, dependent on physical form and particle size. In the proximal stomach near the lower esophageal sphincter, pH ranges from 5.0-7.0 due to the buffering action of bicarbonate provided by saliva from the mouth. Values may drop as low as 2.0-3.0 in the distal stomach that contains dense liquid components of ingesta (Husted et al.,

2009). Hydrochloric acid (HCl) is constantly secreted from the parietal cells and is the major secretory product of the stomach. Production and regulation of HCl involves several physiological feedback loops that respond to intrastomach pH, feed composition, stomach distension, and acetylcholine production. Pepsinogen secreted by chief cells in the stomach wall is converted to its active form, pepsin, upon mixing with HCl in the gastric juice. Pepsin is a proteolytic enzyme that cleaves amide bonds at the C-terminal end, starting the process of protein digestion. Gastric lipase, also secreted by the chief cells, starts the digestion of lipids by breaking down triacylglycerols into diacylglycerols and free fatty acids (Geor et al., 2013).

Chyme from the stomach enters the 25m long SI via the pyloric sphincter. Total transit time through the small intestine ranges from 2-4h, with long stem forages taking longer than chopped or pelleted feeds. Mucosal villi surrounded by crypts line the small intestine; these villi are the site of absorption of nutrients into the bloodstream. Intestinal digestive enzymes are found on microvilli located on the apical surface of epithelial cells on the villi (Geor et al., 2013). Hydrolysable carbohydrates are first digested in the SI by α-amylase from the pancreas, followed by the membrane-bound disaccharidases located in the brush border, including sucrase, maltase, and lactase, that break the disaccharides into monosaccharides that are absorbed across the SI wall by specific transporters (Richardson and Murray, 2016). Fats are emulsified via bile salts from the liver and further broken down by pancreatic lipase, converting diacylglycerols into free fatty acids that can be absorbed into the bloodstream. Trypsinogen, when converted to its active form trypsin by enterokinase produced in the mucosa of the small intestine, starts the process of protein digestion and absorption in the SI. Trypsin is an endopeptidase that hydrolyses basic amino acids and converts all other oligopeptidases from zymogens to their active forms in order to further break down amino acids into absorbable forms (Geor et al., 2013).

The large intestine (LI) accounts for over 60% of the volume of the GIT and is highly developed to process and absorb plant components that are not digestible earlier in the GIT. No mammalian enzymes are produced in the LI. Environmental factors in the LI, including anaerobic conditions and relatively neutral pH levels, are favorable to large populations of anaerobic microorganisms that digest fiber (Geor et al., 2013). Average pH values in the hindgut range from 6.8-7.0 in horses on roughage-based diets and may drop as low as 5.8 in animals fed high-starch diets (Willard et al., 1977). Digesta exiting from the ileum of the SI first enters the cecum, with a volume of 33L, followed by the large colon with a capacity of 80L; these two locations are the primary sites of fermentation. The proximal LI consists of the cecum, right ventral colon (RVC) and left ventral colon (LVC), while the distal LI is made up of the left distal colon (LDC), right distal colon (RDC), small colon (SC) and rectum (Dougal et al., 2013). Upon exiting the large colon, digesta enters the small colon, where the final absorption of water and formation of fecal balls takes place (Geor et al., 2013).

Bacteria represent the major constituent of microbial biomass in the LI. In the cecum, total anaerobes range from $1.85x10^7$ to $2.65x10^9$ cfu/ml of cecal fluid. Cellulolytic bacteria are predominant within the cecum, at $4.6x10^6$ to $9.4x10^6$ cfu/ml. Protozoa are present at levels between 100-1000 cells/ml of fluid, with the highest concentrations found in the left dorsal colon (Fombelle et al., 2003). Defaunation of protozoa in the hindgut of ponies did not affect total or cellulolytic bacteria concentrations and showed no significant effect on cellulose digestibility, while a slight reduction was seen in dry matter digestibility, suggesting that protozoa present in the hindgut do not produce unique enzymes and are not essential for anaerobic fermentation of fiber (Moore and Dehority, 1993).

Composition of the bacterial community shifts depending on anatomical location within the hindgut. Narrowing and folding of the intestine at the pelvic flexure marks the change from proximal to distal, with a concurrent shift in microbial populations. Recent studies have focused on the use of genomic sequencing of 16S rRNA to identify operational taxonomic units (OTUs). Gene sequencing of 16S rRNA forms clusters based on gene similarity that are used to classify bacteria into taxonomic groups (Dougal et al., 2012). Multiple studies have shown that bacterial communities in the GIT exhibit large variation between regions of the GIT and between individual animals, making the identification of a shared core community difficult (Dougal et al., 2012; Dougal et al., 2013). At the phylum level, Firmicutes dominates the hindgut bacterial community, representing 45-80% of all samples, followed by Bacteroidetes at 6-43% and Verrucomicrobia at 0-18% (Shepherd et al., 2012; Steelman et al., 2012; Dougal et al., 2013; Dougal et al., 2014). Large differences in the representation of phylum diversity might be attributed to differences in geographic location, breed, or diet of the animals, or to technique differences in DNA extraction and analysis (Julliand and Grimm, 2016).

Sequencing bacterial genomes past the phylum level is challenging due to the lack of genetic information to form a reference library of genomic information for many genus and species (Julliand and Grimm, 2016). Recent studies have shown that the genus and species of the hindgut microbial community showed high levels of diversity, with approximately 10% of all OTUs forming a core community across all horses sampled. Large numbers of OTUs showed low abundance, with the largest single OTU accounting for only 2% of the sequences in that region of the LI. Eleven taxonomic families made up the entire core community in the RVC and RDC. Significant differences were seen between regions of the LI, with an unclassified family

belonging to the order Bacteroidales showing the highest prevalence in the proximal LI, while *Prevotellaceae* (Bacteroidales) dominated the distal LI (Dougal et al., 2013).

**Effect of Diet on Microbial Population.** Multiple studies have confirmed that changing concentrations of fiber and starch in the diet of horses causes a concurrent shift in the bacterial community present in the GIT. When horses were shifted from a 100% hay diet to an evenly divided hay/barley diet, measured concentrations of total anaerobic, amylolytic, and lactate-utilizing bacteria increased, while a decrease was seen in cellulolytic bacteria (Grimm et al., 2017). On a diet supplemented with either starch-rich grain or fat-rich oil, an increase in proteobacteria was observed when compared to the control diet of hay only (Dougal et al., 2014). Diversity within levels of taxonomic classification is affected by diet; across multiple studies there have been reported reductions in species and family richness when either starch in the form of cereal grains, or fat in form of oils, are added to a forage-only diet (Dougal et al., 2014; Fernandes et al., 2014).

Feeding frequency has been shown to influence microbial communities. In horses fed a high starch concentrate feed either one, two, or three times daily, more frequent meals resulted in lower abundances of *Prevotella spp*., *Lactobacillus spp*., *Strepococcus spp*., and *Coprococcus spp.* In additional to lower overall abundance, *Streptococcus spp.* showed less fluctuations over time in horses fed high starch meals more frequently. At the phylum level, Firmicutes decreased with increased feeding frequency while Bacteroidetes increased with increased feeding frequency (Venable et al., 2017).

**Fiber**. The group of structural carbohydrates from the plant cell wall, generally referred to as fiber, are not a chemically or structurally homogenous group of compounds. Hemicellulose, cellulose, pectin, and lignin are the primary structural components of plant cell walls. Neutral

detergent fiber (NDF) consists of hemicellulose, cellulose, and lignin. Acid detergent fiber (ADF) determinations use an acid solution to remove the hemicellulose from the sample, leaving ADF as a measurement of cellulose and lignin (Van Soest et al., 1991).Hemicellulose, cellulose, and pectin all contain β-1-4 glycosidic bonds that are indigestible by mammalian enzymes. Lignin, an irregularly structured polyphenolic polymer, is included in the fiber group because of its similarities in function within the plant cell wall and its low degradability in the digestive tract (Hartley and Jones, 1977). As plants mature the relative amount of cell wall structures increase while cell contents decrease; this increase in structural components in the plant results in a concurrent decrease in digestibility. Maturation results in increased lignification of the plant cell wall which decreases the availability of the other structural carbohydrates to the microbial populations in the hindgut (Hartley and Jones, 1977).

Equines are less efficient than ruminant species at digesting and utilizing fiber components of plants. Digestibility of ADF has been estimated at 20-40% in horses, with NDF ranging from 35-50% digestible. In comparison, the same forage species when fed to cattle had digestibility estimates of 40-55% for ADF and 40-60% for NDF (Cymbaluk, 1990). The observed differences in digestibility may be due to the faster transit time though the equine GIT and the lower concentration of fiber-degrading bacteria in the equine hindgut when compared to the bovine rumen (Kern et al., 1974).

## Methods of Estimating Digestibility

**Total Fecal Collection.** Conventionally, digestibility of feedstuffs has been quantified with total fecal collection (TFC). Animals are fed the diet of interest and the total output of feces is collected for two to seven days via a collection bag attached to the animal with a harness. Dry

matter digestibility (DMD) is determined as the amount of fecal dry matter divided by feed dry matter. Confinement in pens or stalls is required for the duration of the study period. Constant confinement creates welfare concerns and prevents digestibility studies in pastured, young, or exercising animals (Goachet et al., 2009). Age, diet, and workload have all been shown to effect feed digestibility; therefore, there is a need to conduct digestibility studies across a wide variety of classes of horses (Schaafstra et al., 2018). This method is also labor intensive, requiring each animal's feed to be individually weighed and fed, and feces must be collected and weighed multiple times a day. Because only one diet of interest can be studied at a time, trial periods may became lengthy for studies involving multiple diets, as animals must be adapted to the diet for several weeks before the collection of feces can begin (Goachet et al., 2009).

**Marker Based Studies.** Digestibility can be measured in vivo using the ratio of an indigestible marker present in the feed and feces. Indigestible markers must be unaltered by passage through the gastrointestinal tract, cannot influence physiological processes of digestion, and must be either associated with the nutrient being studied or must pass through the tract at the exact same rate as the nutrient. Markers can be either internal (already present in the feed) or external (separately added to the feed or administered to the animal). Internal markers used for equine digestibility studies include lignin, acid insoluble ash, and n-alkanes. Acid insoluble ash is the most common internal marker used, however the recovered amount of AIA in feces in some studies has exceeded the amount measured in the feedstuff, and the recovery rate of AIA in feces varies based on days fed, impacting accuracy (Sales, 2012). Plant lignin is often not completely recovered in the feces, likely due to microbial digestion in the hindgut of a fraction of lignin, leading to the underestimation of feed digestibility (Goachet et al., 2009; Sales, 2012). Naturally occurring long chain saturated aliphatic hydrocarbons were originally studied as a

marker, however they are consistently recovered at rates lower than one, underestimating feed digestibility (Sales, 2012).

External markers studied primarily include chromic oxide and titanium dioxide. For accuracy in field conditions, a critical feature of viable markers is that the marker must be excreted at a constant rate per unit time. Within day and day-to-day variation in marker recovery in feces has presented challenges with marker-based methods of estimating digestibility (Schaafstra et al., 2018). Chromic oxide provides estimates of digestibility that are generally similar to the TFC method (Sales, 2012). Rate of passage of chromic oxide appears to be affected by the physical form of feed the animal is consuming. When loose long-stem hay was fed with chromic oxide as a marker, digestibility estimates were not different from TFC. Chromic oxide used as a marker with both pelleted and wafer hay resulted in the overestimation of digestibility compared to the control (Haenlein et al., 1966). Diurnal variation in the output of chromic oxide in fecal material also presents a source of inaccuracy, resulting in the need to dose the marker twice a day and collect fecal samples four times per day for the length of the collection period (Holland et al., 1998; Schaafstra et al., 2018). Chromic oxide presents safety concerns for both research animals and researchers as it is a suspected carcinogen (Schaafstra et al., 2018).

Titanium dioxide is an approved food additive that has been recently studied as an alternative to chromic oxide. When compared to TFC, titanium dioxide provided similar estimates of DMD in ponies fed chopped alfalfa hay. Dosage frequencies of both one and two times per day provided fecal recovery levels similar to 100% (Schaafstra et al., 2018). In exercising horses fed a mixed ration of hay and concentrate, titanium dioxide provided estimates of DMD, acid detergent fiber digestibility (ADFD), and neutral detergent fiber digestibility (NDFD) that were similar to those calculated by the TFC technique (Schaafstra et al., 2019).

Marker-based studies present several advantages over TFC. Markers allow for the study of digestibility without the need to confine animals and limit movement for the study period, permitting digestibility trials to be performed in young, exercising, and pastured horses (Schaafstra et al., 2018). However, these techniques still present several challenges. Incomplete or over recovery of markers leads to inaccuracy of digestibility estimates, the need to individually collect fecal samples and dose the marker is labor-intensive and limits study size, and marker-based techniques do not easily allow for the determination of digestibility of individual components of the diet, only for the overall digestibility of the entire combined ration (Haenlein et al., 1966; Holland et al., 1998; Schaafstra et al., 2019).

**In Vitro.** In vitro methods for the determination of forage digestibility provide opportunities for the study of diets that cannot be studied via in vivo digestibility trials (i.e. diets based on low-quality forages that would not meet maintenance nutrient requirements). In vitro batch culture techniques allow for the simultaneous study of multiple feedstuffs and are also less labor intensive than in vivo digestibility trials (Schaafstra et al., 2018). Tilley & Terry (1963) developed a two-step method for determining fiber digestibility that first fermentatively digests feed samples in rumen fluid followed by enzymatic digestion in pepsin. Individual feed samples are placed into test tubes filled with rumen fluid for 48h and agitated by hand four times a day to simulate the mixing action of the rumen; the process is repeated for 24h for pepsin digestion. Other methods use pressure transducers to measure gas production in order to model the kinetics of digestion. Batch culture techniques allow for the simultaneous digestion of multiple feed samples in the same container, allowing for a larger sample size while reducing labor and sources of possible error (Lattimer et al., 2007).

Feedstuffs in ruminant species are first digested fermentatively by microbial populations in the rumen, then further degraded by mammalian chemicals and enzymes in the abomasum and small intestine (Tilley and Terry, 1963). In contrast, monogastric species like the equine first digest feed via mammalian chemicals and enzymes, presenting only the nondigested portions from those processes to the microbial populations in the hindgut (Lowman et al., 1999). Modifying two-stage digestion methods from ruminant research for equine use would suggest the need to first predigest feed to simulate pre-cecal digestion in the equine before using microbial inoculum to fermentatively digest the feed, however research has shown that enzymatic predigestion of feed delayed the onset of fermentation and required longer periods of fermentation to fully digest the feed (Abdouli and Attia, 2007). Enzymatic predigestion of feed for in vitro equine research is necessary to accurately estimate the digestibility of high starch, low fiber feeds such as grains, but not necessary for low quality, high fiber feeds such as hay, which can instead be evaluated using a simpler one-step method using just microbial inoculum (Abdouli and Attia, 2007).

Source of Microbial Inoculum. Traditionally, microbial inoculum for in vitro techniques is formed using rumen fluid from fistulated animals for ruminant studies or using cecal fluid from cannulated animals for monogastric studies. Cecal cannulation is a costly and invasive surgical procedure that requires long recovery periods and has a high mortality risk. Mortality rates of ten percent were observed in two studies of cannulated equines (Peloso et al., 1994; Beard et al., 2011). Cannulated equines are not commonly available due to these problems, and the cannulation of equines for digestibility research presents welfare concerns. An alternative source of microbial inoculum is thus needed for in vitro digestibility research in equines (Earing et al., 2010).

Fecal material provides a readily available and easily collected source of microbial populations from the GIT. Ruminant studies showed that in vitro digestibility measured with microbial inoculum formed from cattle feces gave lower digestibility values than that of traditional rumen fluid inoculum. Lower digestibility estimates are expected, given that the site of microbial fermentation in ruminants is at the start of the digestive tract. Microbes escaping the rumen are largely degraded in the abomasum and SI as protein sources, and it is likely that only a small proportion of the microbial population is able to traverse the GIT unchanged to be excreted in the feces (Akhter et al., 1999). Because equines are hindgut fermenters where microbial digestion is the last stage of digestion before excretion of waste products, fecal microbial populations in equines are likely to be more representative of the GIT population than those from ruminant species.

Lowman et al. (1999) found that equine fecal material was suitable for use as microbial inoculum for in vitro gas production techniques. Freshly collected fecal material was liquidized in anaerobic conditions using carbon dioxide gas and used as inoculum. Gas production profiles for the sixteen feeds studied were typical when compared to earlier results using microbial inoculum formed from cecal fluid. Lattimer et al. (2007) and Earing et al. (2010) both validated the use of microbial inoculum from equine feces for use in the batch culture Daisy II incubator. When results from the Daisy II were compared with in vivo digestibility trial results, using the same animals for both the in vivo and in vitro methods, there were no significant differences between the two methods, showing that the Daisy II system provides valid and accurate estimates of total tract DM digestibility in the equine (Lattimer et al., 2007). Earing et al. (2010) further validated the use of the Daisy II system with equine fecal inoculum, finding no significant differences between the Daisy II and in vivo results across four different diets.

Collection Time. Standardization of inoculum presents a challenge for in vitro digestibility research. Donor animal diet and age, along with inoculum collection time, concentration, and preparation have all been reported as factors that influence in vitro digestibility results (Hervás et al., 2005). Equines evolved as grazing animals that eat small meals frequently through the day. Modern management practices result in horses being kept in confined spaces without access to grazing while being fed 2-3 meals per day. Meal-feeding results in relatively large amounts of feed moving through the digestive tract together, as opposed to the natural condition of a continuous flow of small amounts of feed (Desrousseaux et al., 2012). This change in transit pattern within the digestive tract effects the ability of researchers to use fecal material as inoculum for in vitro digestibility studies.

Desrousseaux et al. (2012) studied the effect of equine fecal sample collection time on in vitro digestibility estimates in a gas production system. Animals were fed on a consistent schedule three times a day and fecal material was collected at four timepoints after the morning feeding: 0, 2, 5, and 8h. Gas production was highest with feces collected 2h after the morning feeding and lowest with feces collected at 0h. Concentrations of VFAs was highest at 0h and tended to be reduced as time after feeding increased. Bacteriological cultures were performed and showed no effect of collection time on the counts of total anaerobic, lactate utilizing, or cellulolytic bacteria.

Preservation Methods. In vitro digestibility studies are typically performed using freshly collected microbial inoculum. Preserved inoculum sources would provide researchers with the ability to use inoculum from animals located further distances from laboratory facilities, to transport inoculum in a variety of temperatures and environmental conditions, and would aid in standardizing in vitro research procedures and results (Murray et al., 2012).

Research in ruminant animals suggests that the fermentation of forages with preserved inoculum is dependent on the forage substrate being fermented. Rumen fluid inoculum from sheep stored on ice at approximately 0°C for 3h or 6h did not show any change in fermentation ability compared to fresh samples. At 24h on ice, gas production rate and extent of feed degradation were reduced ($P<0.05$) when barley straw and alfalfa hay were fermented, but no change was reported for pure starch or cellulose. Freezing inoculum for 24h at -18°C reduced ($P<0.05$) fermentation parameters for all four feeds tested (Hervás et al., 2005). Freezing rumen fluid from cattle at -40°C for 48h resulted in the reduction of amylase activity by 22% and xylanase activity by 52% (Hristov et al., 1999).

Research on the preservation of equine fecal inoculum is limited but supports the conclusion from ruminant studies that the fermentative ability of preserved inoculum is substrate dependent. Freezing equine fecal material at -20°C for 7d reduced ($P<0.05$) in vitro digestibility estimates for grass hay, but did not affect estimates for alfalfa hay, when using a gas production model. Further experiments showed that reducing the freezing time to 24, 48, or 72h still significantly reduced digestibility estimates for grass hay but not for alfalfa hay (Murray et al., 2012).

Cell culture techniques can help elucidate changes in microbial populations caused by preservation methods. Equine fecal material stored for 2h at 37°C showed a 90% reduction in cellulolytic bacteria populations. When stored for 2h at 4°C, there was a >99% reduction in viability of cellulolytic bacteria. Lactobacilli showed a similar response when feces were stored in cold conditions, with reductions in viability when at room temperature or 4°C for 2, 4, or 8h. In contrast, lactobacilli populations in feces remained stable compared to initial counts when stored at 37°C for 2 or 4h. After 8h of storage at 37°C, lactobacilli numbers started to increase,

and after 24h at 37°C, fecal samples had >10-fold more lactobacilli than initial samples (Harlow et al., 2015). The reduction in cellulolytic populations across all storage conditions suggests that the fermentation of high-cellulose feeds will be negatively affected by the use of preserved fecal inoculum, which is in line with earlier work by Murray et al. (2012).

Representativeness of Fecal Material. Culture-dependent and culture-independent techniques have been used to elucidate differences in microbial concentration and diversity in various sections of the LI (Dougal et al., 2013; Harlow et al., 2015). Because of welfare and practical concerns with cecum and colon cannulation, fecal material is often used to study the hindgut environment of the horse, both for direct microbial assessment and as inoculum for in vitro digestibility studies. Individual compartments of the hindgut represent individual microbiomes, with differences in environment that may affect microbial populations, therefore it is important to determine whether fecal material is representative of the entire hindgut microbiome (Dougal et al., 2012; Julliand and Grimm, 2016).

Quantifications of total bacteria in the hindgut at three locations showed higher concentrations of bacterial DNA per gram of digesta in the cecum as compared to the RDC or feces, whilst the RDC and feces had similar concentrations of bacterial DNA. Protozoal rDNA concentrations were similar in the cecum and feces and significantly higher in the RDC, whereas anaerobic fungi were present at similar levels in all three measured locations (Dougal et al., 2012). Further work by Dougal et al. (2013) that sampled seven locations in the LI confirmed the previous work, finding that the bacterial profile of fecal material was similar to that of the distal LI but significantly different from the proximal LI. Proximal sections of the LI show greater species diversity and richness compared to distal regions and feces.

Other studies have shown contradictory results. One such study found that there were significantly fewer bacteria in the cecum than in the dorsal colon and rectum, which had similar levels. However, this study evaluated only *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Streptococcus bovis* (Hastie et al., 2008). Given the wide range of bacterial species present in the hindgut as indicated by other studies, it is likely that evaluating only three species did not provide an accurate estimation of total bacterial levels (Dougal et al., 2013; Fernandes et al., 2014; Julliand and Grimm, 2016). Sadet-Bourgeteau et al. (2014) found no difference in microbial populations between the cecum and colon, however the single site of colon sampling was the RVC, which directly follows the cecum in the proximal LI. Significant differences were seen when fecal material from the most distal section of the LI was compared to the two most proximal sections of the LI, the cecum and RVC. Previous work by Dougal et al. (2012; 2013) has shown that there are similarities in microbial communities within different individual parts of the proximal and distal LI, with differences only observed when comparing sections of the proximal LI to sections of the distal LI.

Storage Temperature. Gram staining allows for the differentiation of bacteria into broad groups by the characteristics of their cell walls. Gram-positive (G+) bacteria possess a thick cross-linked peptidoglycan layer in the cell wall (20-80nm) with no lipid-based outer membrane, while gram-negative (G-) bacteria have only a thin peptidoglycan layer (2-3nm) with an outer lipid membrane. Structural differences in bacterial cell walls between taxa have important consequences for bacterial survival in various environmental conditions. Cross-linking of peptidoglycan in the cell wall provides rigidity that allows G+ bacteria a greater ability to resist cellular destruction from freezing compared to G- bacteria (Sutcliffe, 2010). Miyamoto-Shinohara et al. (2000) showed that across a variety of genus and species, G+ bacteria survived

freezing at a rate of 80%, whereas only 50% of G- bacteria survived. Freezing temperature appears to influence bacterial survival rates; in samples taken from the swine GIT, significantly higher proportions of bacterial DNA were degraded at -80°C as compared to -20°C (Mølbak et al., 2006).

Freeze-induced injuries and death are due to several mechanisms that are not all fully understood. Some cells, upon exposure to freezing temperatures, never regain the ability to divide and multiply and are effectively dead. Other cells have reduced abilities to grow, due to leakage of cellular contents, greater susceptibility to naturally occurring surfactants, increased nutritional needs, and extended lag periods. Often bacteria that survive freezing do not exhibit apparent structural damage but do show increased lag times before they start to multiply at the same rate as controls, likely due to time needed to repair cellular molecular damage from freezing. Frozen *E. coli* bacteria had a 3-4h lag time, compared to *E. coli* at 25°C and 35°C that had 2 and 1h lag times, respectively (Ray and Speck, 1973). There are many proposed models for the mechanisms by which freezing damages bacterial cells. Cell membrane damage from several root causes can physically or chemically alter the cell membrane, increasing permeability. Ice crystals, especially those formed intracellularly, can physically destroy cell walls. Freezing of water both intracellularly and extracellularly can increase the concentration of solutes present in the cell, influencing turgor pressure within cells. Enzyme activity is temperature-specific, and freezing can both slow down and speed up various metabolic processes within the cell (Ray and Speck, 1973; Pembrey et al., 1999).

Both fiber-utilizing and starch-utilizing bacteria reside in the equine hindgut. Generally larger amounts of fiber-utilizing bacteria are found in the proximal sections of the hindgut, while larger proportions of starch-utilizing bacteria are found in the distal portions, owing to the longer

residence time of high fiber feeds in the cecum (Hastie et al., 2008). Firmicutes (45-80%) and fibrobacter (2-7%) represent the primary cellulolytic phyla in the hindgut (Julliand et al., 1999; Daly et al., 2001; Dougal et al., 2014). *Fibrobacter succinogenes* from the fibrobactar phylum is a G- bacterium that is the primary cellulolytic species in the hindgut, representing 12% of the hindgut bacteria. *Ruminococcus flavefaciens*, a G+ cocci from the Firmicutes phylum, represents 4% of the bacteria found in the cecum/colon (Shepherd et al., 2014). Various species within the Bacteroides genus, a group of G- bacillus organisms (phylum Bacteroidetes), have also been shown to have cellulose-degrading properties. There are a wide variety of starch-utilizing bacteria present within the hindgut, with 95% of amylolytic species being G+ according to one study (Harlow et al., 2016). *Lactobacillus spp, streptococcus spp*, *enterococcus spp*., and *lachnospiraceae spp*. are all are G+ starch-utilizing bacteria found in the hindgut of the horse.

The relatively higher proportion of G- bacteria within the cellulolytic group and higher proportion of G+ species within the amylolytic group provides an avenue for understanding the earlier discussion about the substrate-dependent changes in digestibility when frozen inoculum is used. Because G+ bacteria are more resistant to cellular damage from freezing, these bacteria within the microbial inoculum are more likely to survive freezing, whereas the G- cellulolytic species are more likely to be damaged, explaining the reduction in digestibility seen for highly fibrous feeds like low-quality hay (Ray and Speck, 1973; Julliand and Grimm, 2016).

Diet. The previously discussed research on diet and microbial communities suggests that for in vitro studies of equine digestibility, microbial inoculum should be formed used fecal samples from animals maintained on similar diets to those of interest (Lowman et al., 1999; Julliand and Grimm, 2016). Because microbial communities change in response to the diet being fed, in vitro testing of diets dissimilar to those being fed to the microbial inoculum donors may

produce inaccurate results. If the diets being studied in vitro are based on low-quality forage that is low in soluble carbohydrates and high in ADF components, microbial inoculum should be formed from animals maintained on a diet of similar hay. Likewise, using microbial inoculum from mature, idle animals maintained on a maintenance diet of low-quality hay will likely underestimate digestibility of high-quality forages, as the microbial population from these animals are not adapted to an easily fermentable diet (Dougal et al., 2013; Dougal et al., 2014).

## METHODS AND MATERALS

Three mature quarter horse geldings were selected from the herd at Missouri State University. Animals ranged from 7-19 years of age. All procedures involving the care, management, and use of horses in this study were approved by the Institutional Animal Care and Use Committee on September 13, 2019 (#19-021). Two animals were housed in 3.6x7.3m covered pens on limestone footing; one was housed in a 35x35m covered arena on sand. Animals were fed twice a day at 0700 and 1700 and maintained on their normal rations of 3.6kg per day of a commercial grain mix (Easy Keeper Edge, MFA Inc., Columbia, MO) and 7.3 kg per day of locally produced orchard grass hay with ad libitum access to water.

### Fecal Samples

Fecal samples were collected at 1100 hours from all three geldings via rectal palpation. Samples were placed into sealed plastic bags with the air removed and stored in a prewarmed cooler at 39°C until they reached the laboratory to be processed (5-10 minutes). In the laboratory pooled fecal samples were formed by weighing out 15g of fecal material from each horse and combining them for a pooled sample of 45g. After weighing and pooling four samples, each pooled sample was placed into an unsealed plastic bag with the top left open to provide aerobic conditions, apart from the 39°C sample, which was sealed until further processing.

### Forage Samples

Six different forage samples representing a variety of chemical compositions were used. Forage analysis results are presented in Table 1. Representative samples were taken from each of

five bales of hay and one bag of alfalfa cubes. Samples were ground to pass through a 1mm screen using a Wiley mill and were stored in sealed plastic bags. Dry matter of each forage was determined in the laboratory by drying forage samples for 24h in a 50±2°C oven. Dry matter was calculated as (dry sample weight (g) / starting sample weight (g)) x 100 = percent dry matter.

F57 filter bags (Ankom Technology) were used within the Daisy II incubator for the calculation of forage digestibility. Filter bags were first immersed in acetone for five minutes and air dried on a wire screen. Empty filter bags were labeled and weighed. Labels included identification of date, treatment, and forage sample. Every forage sample was duplicated within individual digestion jars and the results were averaged for more accurate estimation of DMD. Two filter bags were filled with approximately 0.50g of each forage sample and final weights were recorded before sealing the bags with a 120V Impulse Heat Sealer (American International Electric). One blank bag without any forage was also weighed and sealed to allow for adjustment for forage particle attachment in the digestion jars. Each digestion jar contained duplicate filter bags filled with each of six forages, plus one blank filter bag (n=13).

**Storage Conditions**

Four storage temperatures were evaluated. One sample was kept at 39°C, processed quickly, and immediately used to form inoculum. One each of the other three samples was left at 22°C for 6h, 3°C for 6h, or -18°C for 24h to simulate a variety of storage and environment conditions. The 22°C sample was stored on the counter in a climate-controlled laboratory; the 3°C sample was placed in a standard household refrigerator in the laboratory; and the -18°C sample was placed in a standard household freezer in the laboratory. To form inoculum, each 45g pooled fecal sample was individually placed in an Oster blender containing 400mL of

distilled water, purged with CO2 for 15 seconds, and blended for 15 seconds. The inoculum was strained through four layers of cheesecloth into a beaker to remove solid particles. Strained inoculum was poured into Ankom digestion jars containing filter bags with forage samples and 1600mL of buffer solution for a total of 2000mL of mixed inoculum and buffer. Digestion jars were purged with CO2 for 30 seconds before being closed and placed into the Ankom Daisy II Incubator. Each digestion jar within the Daisy II Incubator contained fecal inoculum prepared from one of the four temperature conditions.

Buffer solution was made by combining 1333mL of solution A ($KH_2PO_4$ 10.0g/L; $MgSO_4 \cdot 7\ H_2O$ 0.5g/L; NaCl 0.5g/L; $CaCl_2 \cdot 2\ H_2O$ 0.1g/L; $CH_4N_2O$ 0.5g/L) with 267mL of solution B ($Na_2CO_3$ 15.0g/L; $Na_2S \cdot 7\ H_2O$ 1.0g/L) at 39°C and titrating to a pH of 6.8. After mixing, 1600mL of buffer solution was added to each digestion jar and allowed to equilibrate at 39°C in the Daisy II Incubator for at least 30 minutes while fecal inoculum was being collected and prepared.

**Sample Analysis**

Once placed into the Daisy II incubator, individual digestion jars were incubated for 48h. After removal from the incubator, filter bags were rinsed in cool tap water until the water ran clear to ensure all fecal inoculum was removed and that enzymatic digestion of the sample was stopped. Once rinsed, filter bags were placed into an oven at 50±2°C for 24h to dry and then the filter bags were cooled to room temperature in an Ankom MoistureStop Weigh Pouch (Ankom X45, Ankom Technology) before being individually removed and reweighed to determine DMD. Dry matter digestibility was determined as [(100-(final dry weight-(initial bag weight x correction factor))) ÷ (initial sample weight x DM)] x 100 = DMD.

The Ankom Fiber Analyzer (ANKOM$^{2000}$, Ankom Technology, Mecedon, NY) was used to determine digestibility of the NDF and ADF fiber fractions as outlined by Ankom Technology. Briefly, concentrated NDF solution and triethlene glycol (Ankom FND20C) was diluted with 20L of distilled water and added to an Ankom Cubitainer attached to the Ankom Fiber Analyzer. The dried filter bags from DMD determination were added to the fiber analyzer chamber. 20g of sodium sulfite and 4ml of a-amylase were added to the chamber containing forage samples, and another 8ml of a-amylase was added to the automatic dispenser for the rinse cycles. After cycle completion, the filter bags were soaked in acetone for 5 minutes, air dried on a wire screen until evaporation of acetone was complete, then dried in a 100±2°C oven for 2-4h. Samples were placed in an Ankom MoistureStop Weigh Pouch (Ankom X45, Ankom Technology) to cool to room temperature, and then individually removed and weighed to allow for the calculation of NDFD.

Analysis of ADFD was completed in a similar fashion. Concentrated ADF solution (Ankom FAD20) was diluted with 20L of distilled water and poured into an Ankom Cubitaner and attached to the Fiber Analyzer. Filter bags from the NDFD determination were placed into the chamber and then removed and dried as described above. Dry weights were measured and ADFD was determined.

**Statistical Analysis**

Individual jars in the Daisy II Incubator contained fecal inoculum from one of the four treatment conditions along with duplicates of six forage samples. Means of duplicate samples were calculated and used for analysis. Three separate runs were conducted over a two-week period, giving n=3 measurements for each forage sample under each treatment condition, and

n=18 total measurements for each treatment. Data were analyzed using SAS. A mixed linear model was used to analyze differences in DMD, NDFD, and ADFD, with forage and treatment as class statements with date included as a random effect to account for natural variability in digestibility over time. No significant interaction of forage x treatment was observed (P=0.81 for DMD). Tukey's Honest Significant Difference test was used to make pairwise comparisons to determine which treatments showed significant differences. Further analysis with a mixed linear model was used to evaluate the effect of forage quality parameters CP, NDF, and ADF as covariates for DMD, NDFD, and ADFD results.

Table 1. Forage sample chemical composition and nutrient analysis of dry matter, neutral detergent fiber, acid detergent fiber and crude protein (DM basis).

| | Forage Chemical Composition | | | |
|---|---|---|---|---|
| Forage Sample | % DM | % NDF | % ADF | % CP |
| Alfalfa Cubes | 91.59 | 40.75 | 32.15 | 19.88 |
| Alfalfa Hay | 92.01 | 45.71 | 31.93 | 19.60 |
| Alfalfa Mix Hay | 91.53 | 61.27 | 42.71 | 11.57 |
| Fescue Hay 1 | 91.24 | 61.69 | 44.67 | 5.97 |
| Fescue Hay 2 | 91.22 | 62.40 | 45.05 | 9.26 |
| Orchard Grass Hay | 91.59 | 53.85 | 40.29 | 13.76 |

Forage chemical composition parameters measured using NIR spectrometry for crude protein (CP) and wet chemistry for neutral detergent fiber (NDF) and acid detergent fiber (ADF). Dry matter (DM) measured in a 50±2°C oven.

# RESULTS

## Digestibility

All three digestibility measures used (% DMD, % NDFD, and % ADFD) declined as the percent NDF and ADF of the forage increased (Figure 1 and Table 2).

Means of DMD of digested forage samples were analyzed by treatment using a mixed linear model and post-hoc Tukey testing (Figure 1). Significant differences ($P<0.0001$) were observed between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were found between pairwise comparisons for 39°C and 22°C or 3°C and -18°C (Table 3).

When NDFD was analyzed, significant differences ($P<0.01$) were observed between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were found between pairwise comparisons for 39°C and 22°C or 3°C and -18°C (Figure 2 and Table 4).

Similar results were seen for ADFD, with significant differences ($P<0.001$) between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were observed between pairwise comparisons for 39°C and 22°C or 3°C and -18°C (Figure 3 and Table 5).

## Covariates for Dry Matter Digestibility

A mixed linear model was used to evaluate three forage composition parameters (NDF, ADF, and CP) as covariates for DMD by treatment. Four linear regressions were modeled, one for each treatment, for each analysis of covariance. For each covariate, slopes were compared

between all four treatments; if no significant difference in slope was found then models were refitted without the interaction term of covariate x treatment and y-intercepts were compared. No difference ($P>0.05$) in slope was found between the four treatment levels when CP, NDF, or ADF was modeled as a covariate. When refitted without the interaction term, significant differences ($P<0.05$) were observed between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were found between pairwise comparisons of 39°C and 22°C or 3°C and -18°C. Individual covariate graphs for DMD by NDF, ADF, and CP are presented respectively in Figures 4, 5, and 6, with pairwise comparison results presented in Tables 6, 7, and 8.

**Covariates for Neutral Detergent Fiber Digestibility**

A mixed linear model was used to evaluate three forage composition parameters (CP, NDF, and ADF) as covariates for NDFD by treatment. Four linear regressions were modeled, one for each treatment, for each analysis of covariance. For each covariate, slopes were compared between all four treatments; if no significant difference in slope was found then models were refitted without the interaction term of covariate x treatment and y-intercepts were compared. No difference ($P>0.05$) in slope was found between the four treatment levels when CP, NDF, or ADF was modeled as a covariate. When refitted without the interaction term, significant differences ($P<0.05$) were observed between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were found between pairwise comparisons for 39°C and 22°C or 3°C and -18°C. Individual covariate graphs for NDFD by NDF, ADF, and CP are presented respectively in Figures 7, 8, and 9, with pairwise comparison results presented in Tables 9, 10, and 11.

**Covariates for Acid Detergent Fiber Digestibility**

A mixed linear model was used to evaluate three forage composition parameters (CP, NDF, and ADF) as covariates for ADFD by treatment. Four linear regressions were modeled, one for each treatment, for each analysis of covariance. For each covariate, slopes were compared between all four treatments; if no significant difference in slope was found then models were refitted without the interaction term of covariate x treatment and y-intercepts were compared. No difference ($P>0.05$) in slope was found between the four treatment levels when CP, NDF, or ADF was modeled as a covariate. Without the interaction term, significant differences ($P<0.05$) were observed between pairwise comparisons of 39°C and 3°C; 39°C and -18°C; 22°C and 3°C; and 22°C and -18°C. No differences ($P>0.05$) were found between pairwise comparisons of 39°C and 22°C or 3°C and -18°C. Individual covariate graphs for ADFD by NDF, ADF, and CP are presented respectively in Figures 10, 11, and 12, with pairwise comparison results presented in Tables 12, 13, and 14.

Table 2. Average percent dry matter digestibility (% DMD), percent neutral detergent fiber digestibility (% NDFD), and percent acid detergent fiber digestibility (% ADFD) by forage and microbial inoculum storage temperature.

| | | Digestibility Measurements | | |
|---|---|---|---|---|
| Forage | Temperature | % DMD | % NDFD | % ADFD |
| AC | 39°C | 54.4±1.81 | 39.8±0.93 | 38.8±0.44 |
| AC | 22°C | 54.6±1.07 | 43.8±1.03 | 41.9±1.31 |
| AC | 3°C | 50.2±1.20 | 36.5±0.50 | 34.3±0.31 |
| AC | -18°C | 49.2±1.80 | 39.5±1.06 | 38.1±1.10 |
| AH | 39°C | 56.8±1.48 | 41.0±1.78 | 38.9±1.27 |
| AH | 22°C | 55.9±0.83 | 41.6±1.41 | 37.7±0.91 |
| AH | 3°C | 53.2±1.51 | 37.8±1.17 | 34.0±1.19 |
| AH | -18°C | 52.1±1.30 | 37.0±1.08 | 33.8±1.06 |
| AM | 39°C | 32.8±1.47 | 25.6±1.43 | 24.1±1.09 |
| AM | 22°C | 33.2±0.81 | 24.7±0.91 | 22.2±0.89 |
| AM | 3°C | 28.0±1.16 | 25.3±0.20 | 22.8±0.08 |
| AM | -18°C | 28.1±0.93 | 24.0±0.52 | 21.3±0.48 |
| F1 | 39°C | 27.8±1.04 | 25.0±2.32 | 22.8±1.30 |
| F1 | 22°C | 27.6±1.16 | 25.0±1.07 | 23.7±0.59 |
| F1 | 3°C | 24.2±1.38 | 21.1±1.55 | 19.1±1.47 |
| F1 | -18°C | 24.2±1.07 | 21.0±1.67 | 19.1±1.66 |
| F2 | 39°C | 30.1±1.87 | 25.3±0.92 | 23.9±0.86 |
| F2 | 22°C | 27.0±1.97 | 23.6±1.40 | 22.6±0.64 |
| F2 | 3°C | 25.2±0.97 | 20.2±0.48 | 19.2±0.75 |
| F2 | -18°C | 25.6±0.44 | 20.4±1.29 | 19.3±1.18 |
| OG | 39°C | 40.1±1.30 | 33.5±0.55 | 32.5±1.07 |
| OG | 22°C | 39.6±1.40 | 32.8±1.92 | 31.1±1.76 |
| OG | 3°C | 36.4±1.64 | 30.3±1.49 | 27.9±1.64 |
| OG | -18°C | 37.6±1.01 | 31.3±1.54 | 29.8±0.78 |

Values presented as mean ± standard error of the mean. AC = Alfalfa Cubes; AH = Alfalfa Hay; AM = Alfalfa Mix; F1 = Fescue Hay 1; F2 = Fescue Hay 2; OG = Orchard Grass Hay

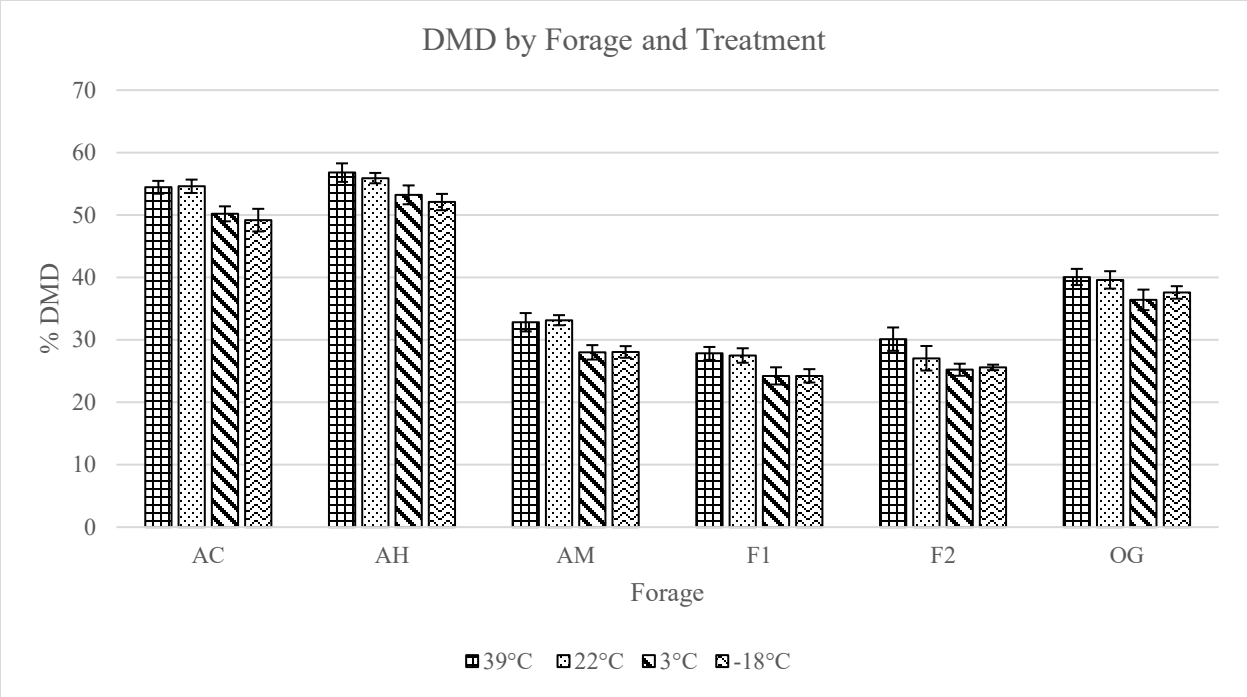


Figure 1. Equine dry matter digestibility of hay by microbial inoculum storage temperature, grouped by forage sample. AC = Alfalfa Cubes; AH = Alfalfa Hay; AM = Alfalfa Mix; F1 = Fescue Hay 1; F2 = Fescue Hay 2; OG = Orchard Grass Hay

Table 3. Difference of least squares means for equine dry matter digestibility of hay by microbial inoculum storage temperature with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.7097 | 0.7468 | 0.7779 |
| 39°C | 3°C | 4.1330 | 0.7468 | <0.0001* |
| 39°C | -18°C | 4.2215 | 0.7468 | <0.0001* |
| 22°C | 3°C | 3.4233 | 0.7468 | 0.0002* |
| 22°C | -18°C | 3.5118 | 0.7468 | 0.0001* |
| 3°C | -18°C | -0.0886 | 0.7468 | 0.9994 |

* indicates significant p-value (P<0.05).

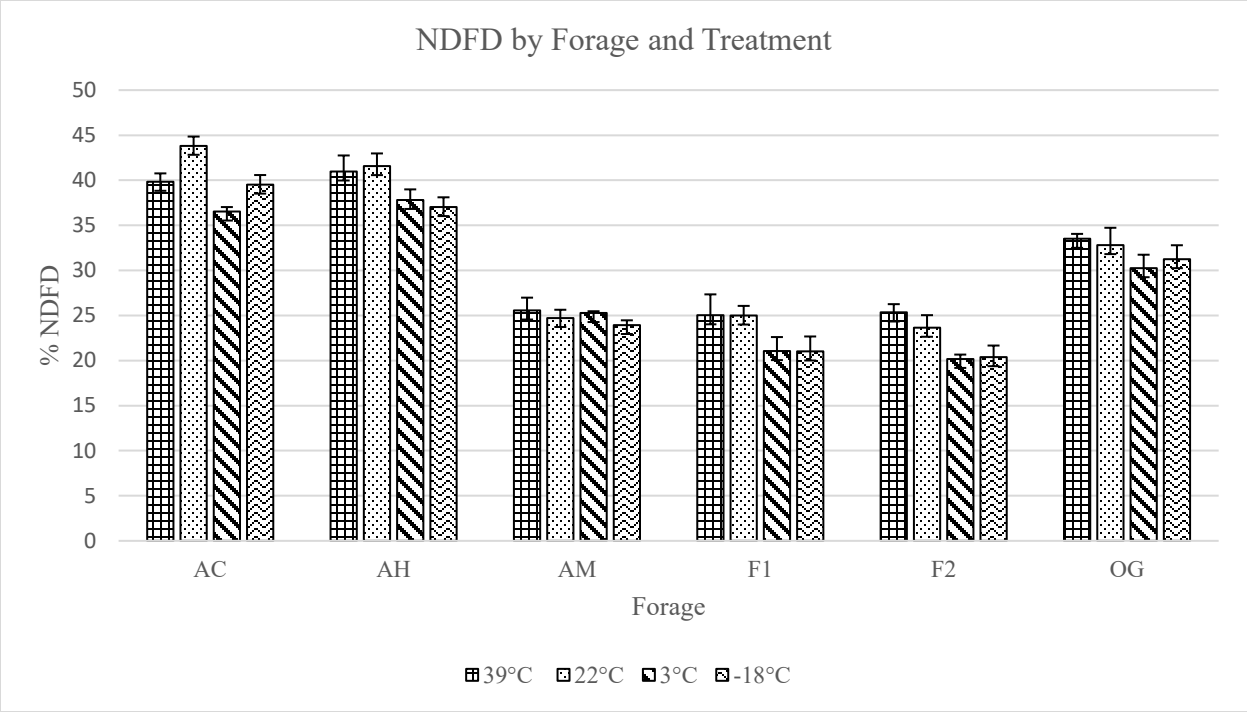


Figure 2. Equine neutral detergent fiber digestibility of hay by microbial inoculum storage temperature, grouped by forage sample. AC = Alfalfa Cubes; AH = Alfalfa Hay; AM = Alfalfa Mix; F1 = Fescue Hay 1; F2 = Fescue Hay 2; OG = Orchard Grass Hay

Table 4. Difference of least squares means for equine neutral detergent fiber digestibility of hay by microbial inoculum storage temperature with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.2245 | 0.7372 | 0.9901 |
| 39°C | 3°C | 3.1861 | 0.7372 | 0.0004* |
| 39°C | -18°C | 2.8480 | 0.7372 | 0.0018* |
| 22°C | 3°C | 3.4106 | 0.7372 | 0.0002* |
| 22°C | -18°C | 3.0725 | 0.7372 | 0.0007* |
| 3°C | -18°C | -0.3381 | 0.7372 | 0.9676 |

* indicates significant p-value (P<0.05).

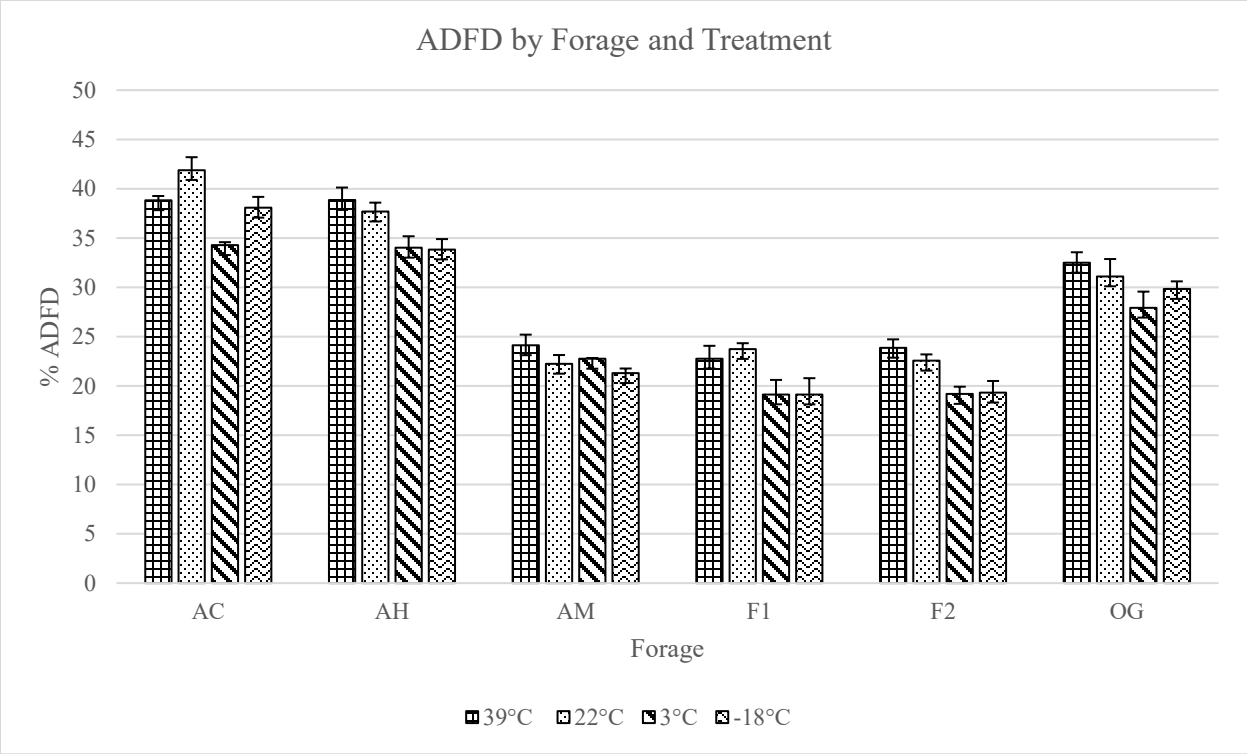


Figure 3. Equine acid detergent fiber digestibility of hay by microbial inoculum storage temperature, grouped by forage sample. AC = Alfalfa Cubes; AH = Alfalfa Hay; AM = Alfalfa Mix; F1 = Fescue Hay 1; F2 = Fescue Hay 2; OG = Orchard Grass Hay

Table 5. Difference of least squares means for equine acid detergent fiber digestibility of hay by microbial inoculum storage temperature with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.2830 | 0.6233 | 0.9685 |
| 39°C | 3°C | 3.9402 | 0.6233 | <0.0001* |
| 39°C | -18°C | 3.2422 | 0.6233 | <0.0001* |
| 22°C | 3°C | 3.6572 | 0.6233 | <0.0001* |
| 22°C | -18°C | 2.9592 | 0.6233 | 0.0001* |
| 3°C | -18°C | -0.6980 | 0.6233 | 0.6792 |

* indicates significant p-value (P<0.05).

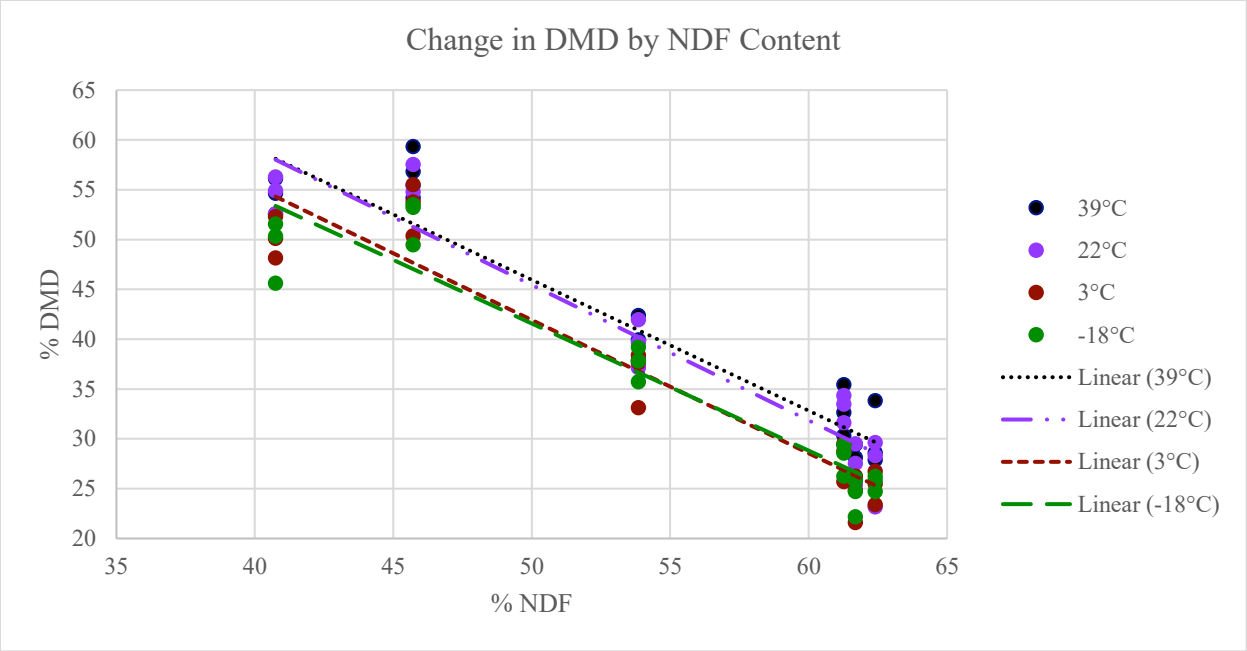


Figure 4. Microbial inoculum storage temperature by neutral detergent fiber interaction in equine dry matter digestibility of hay regression analysis of covariance.

Table 6. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine dry matter digestibility of hay with neutral detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.7097 | 1.1951 | 0.9336 |
| 39°C | 3°C | 4.1330 | 1.1951 | 0.0051* |
| 39°C | -18°C | 4.2215 | 1.1951 | 0.0041* |
| 22°C | 3°C | 3.4233 | 1.1951 | 0.0279* |
| 22°C | -18°C | 3.5118 | 1.1951 | 0.0229* |
| 3°C | -18°C | -0.0886 | 1.1951 | 0.9999 |

* indicates significant P-value (P<0.05).

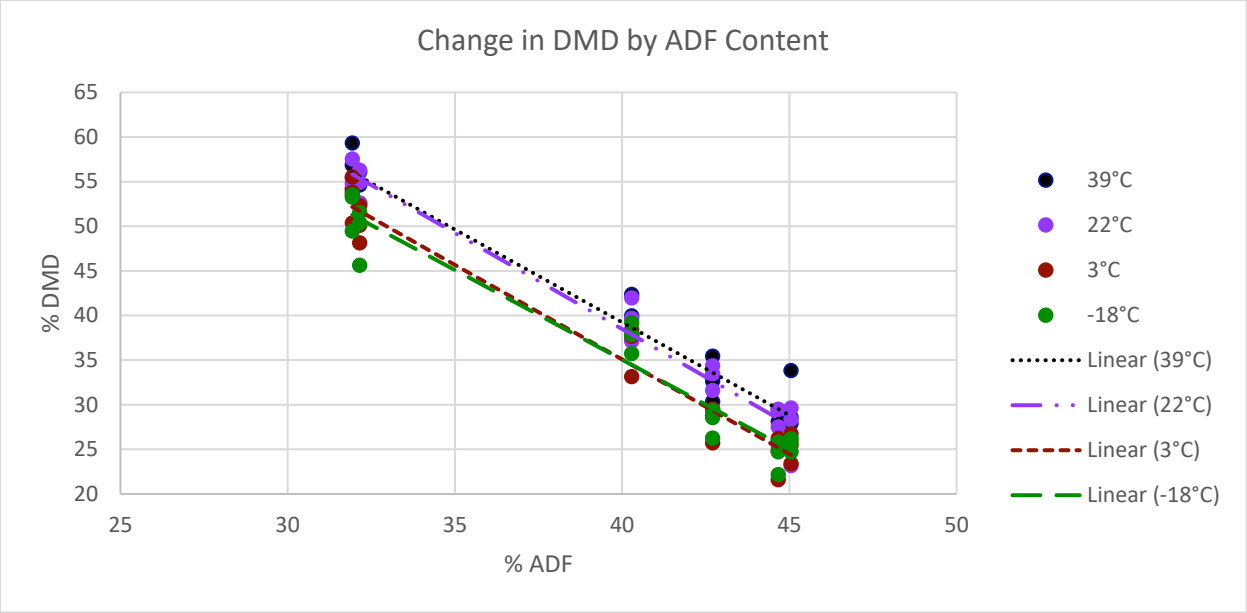


Figure 5. Microbial inoculum storage temperature by acid detergent fiber interaction in equine dry matter digestibility of hay regression analysis of covariance.

Table 7. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine dry matter digestibility of hay with acid detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.7097 | 0.7897 | 0.8055 |
| 39°C | 3°C | 4.1330 | 0.7897 | <0.0001* |
| 39°C | -18°C | 4.2215 | 0.7897 | <0.0001* |
| 22°C | 3°C | 3.4233 | 0.7897 | 0.0003* |
| 22°C | -18°C | 3.5118 | 0.7897 | 0.0002* |
| 3°C | -18°C | -0.0886 | 0.7897 | 0.9995 |

* indicates significant p-value (P<0.05).

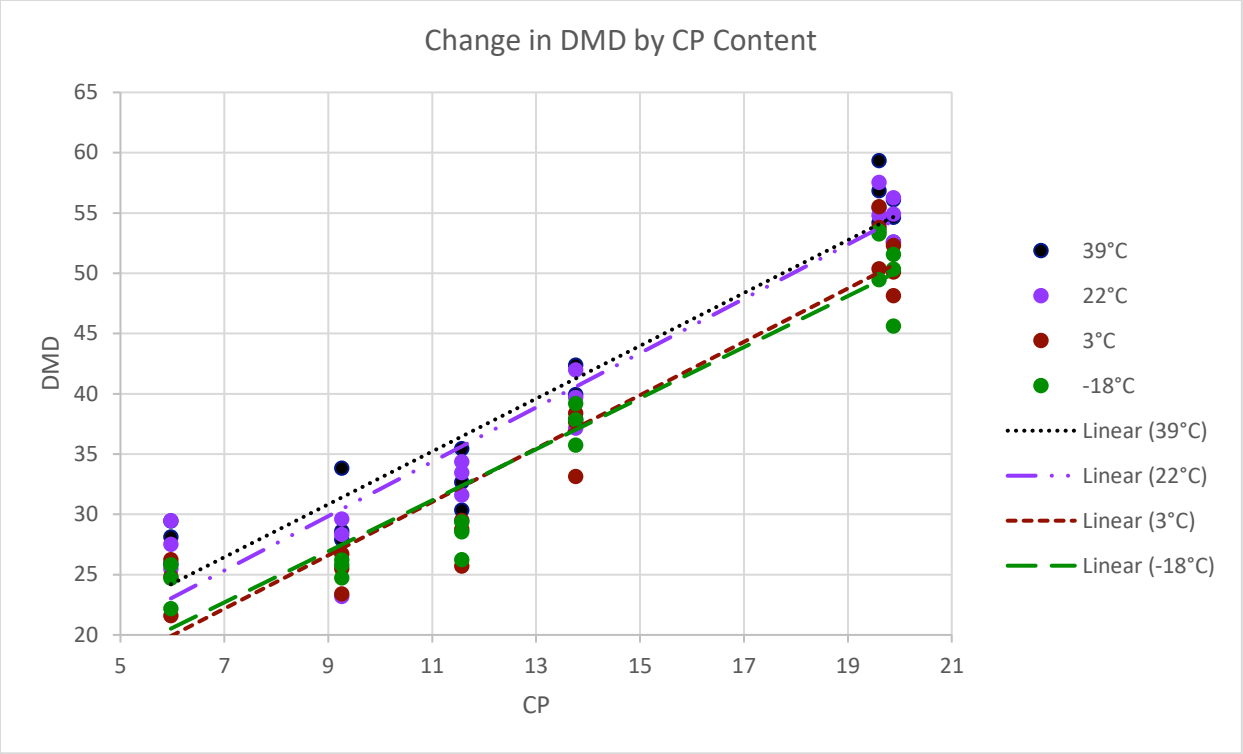


Figure 6. Microbial inoculum storage temperature by crude protein interaction in equine dry matter digestibility of hay regression analysis of covariance.

Table 8. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine dry matter digestibility of hay with crude protein as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.7097 | 1.1338 | 0.9234 |
| 39°C | 3°C | 4.1330 | 1.1338 | 0.0029* |
| 39°C | -18°C | 4.2215 | 1.1338 | 0.0022* |
| 22°C | 3°C | 3.4233 | 1.1338 | 0.0184* |
| 22°C | -18°C | 3.5118 | 1.1338 | 0.0148* |
| 3°C | -18°C | -0.0886 | 1.1338 | 0.9998 |

* indicates significant p-value (P<0.05).

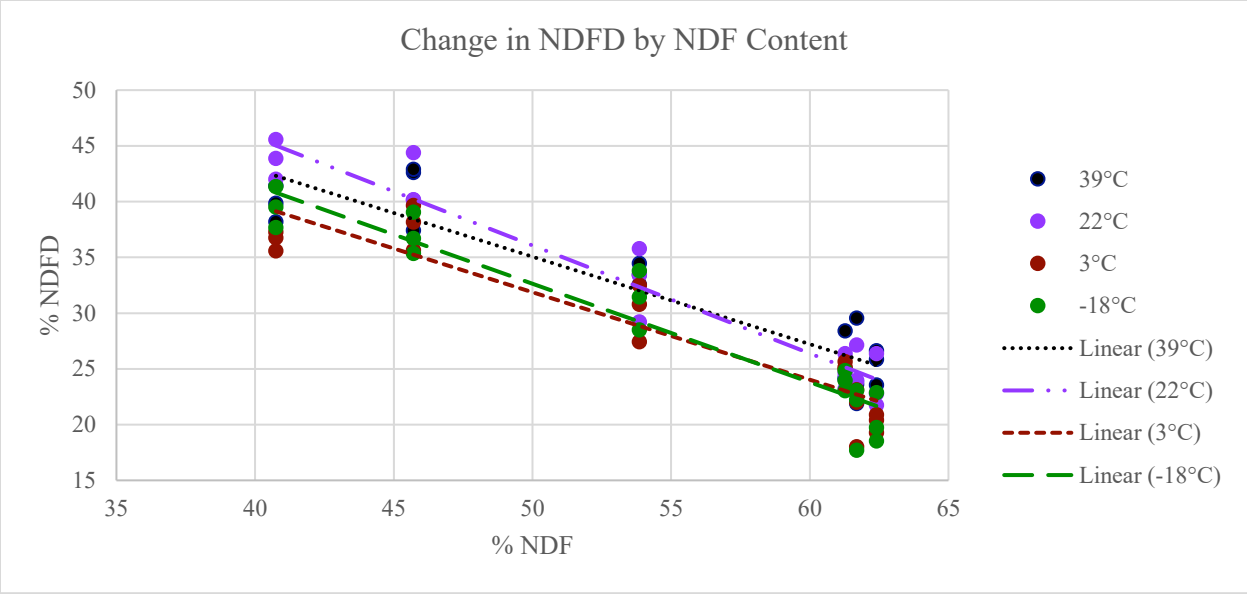


Figure 7. Microbial inoculum storage temperature by neutral detergent fiber interaction in equine neutral detergent fiber digestibility of hay regression analysis of covariance.

Table 9. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine neutral detergent fiber digestibility of hay with neutral detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.5053 | 0.7515 | 0.9072 |
| 39°C | 3°C | 3.1749 | 0.7515 | 0.0004* |
| 39°C | -18°C | 3.7422 | 0.7515 | <0.0001* |
| 22°C | 3°C | 2.6696 | 0.7515 | 0.0038* |
| 22°C | -18°C | 3.2370 | 0.7515 | 0.0003* |
| 3°C | -18°C | -0.5674 | 0.7515 | 0.8742 |

* indicates significant p-value (P<0.05).

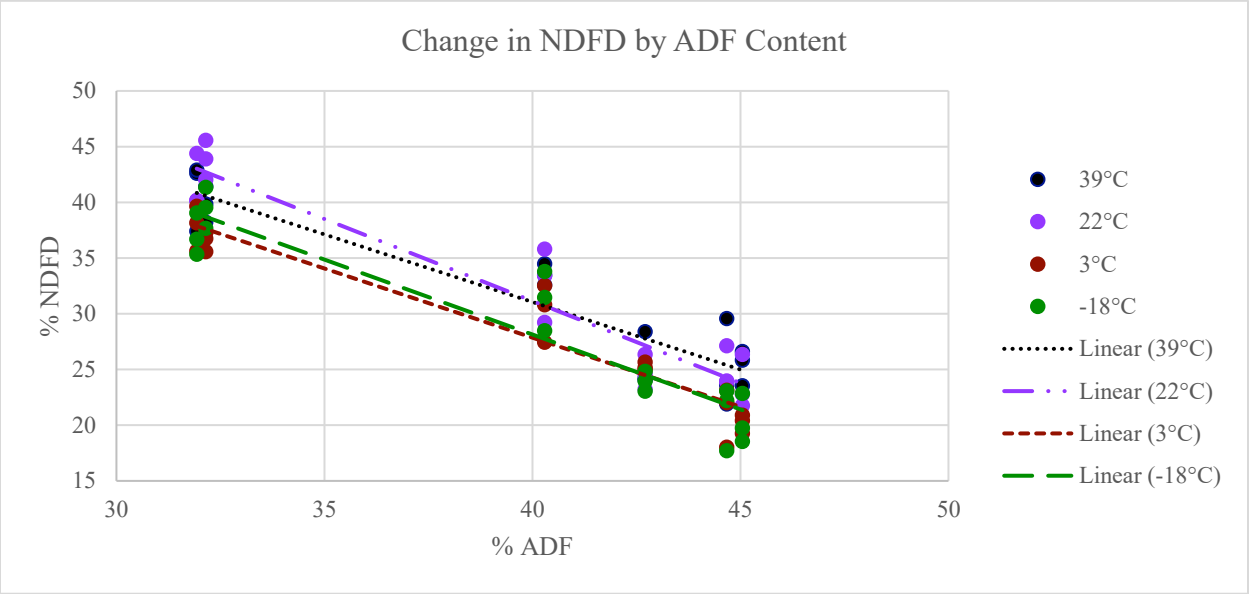


Figure 8. Microbial inoculum storage temperature by acid detergent fiber interaction in equine neutral detergent fiber digestibility of hay regression analysis of covariance.

Table 10. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine neutral detergent fiber digestibility of hay with acid detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.5053 | 0.9127 | 0.9453 |
| 39°C | 3°C | 3.1749 | 0.9127 | 0.0048* |
| 39°C | -18°C | 3.7422 | 0.9127 | 0.0006* |
| 22°C | 3°C | 2.6696 | 0.9127 | 0.0237* |
| 22°C | -18°C | 3.2370 | 0.9127 | 0.0039* |
| 3°C | -18°C | -0.5674 | 0.9127 | 0.9248 |

* indicates significant p-value (P<0.05).

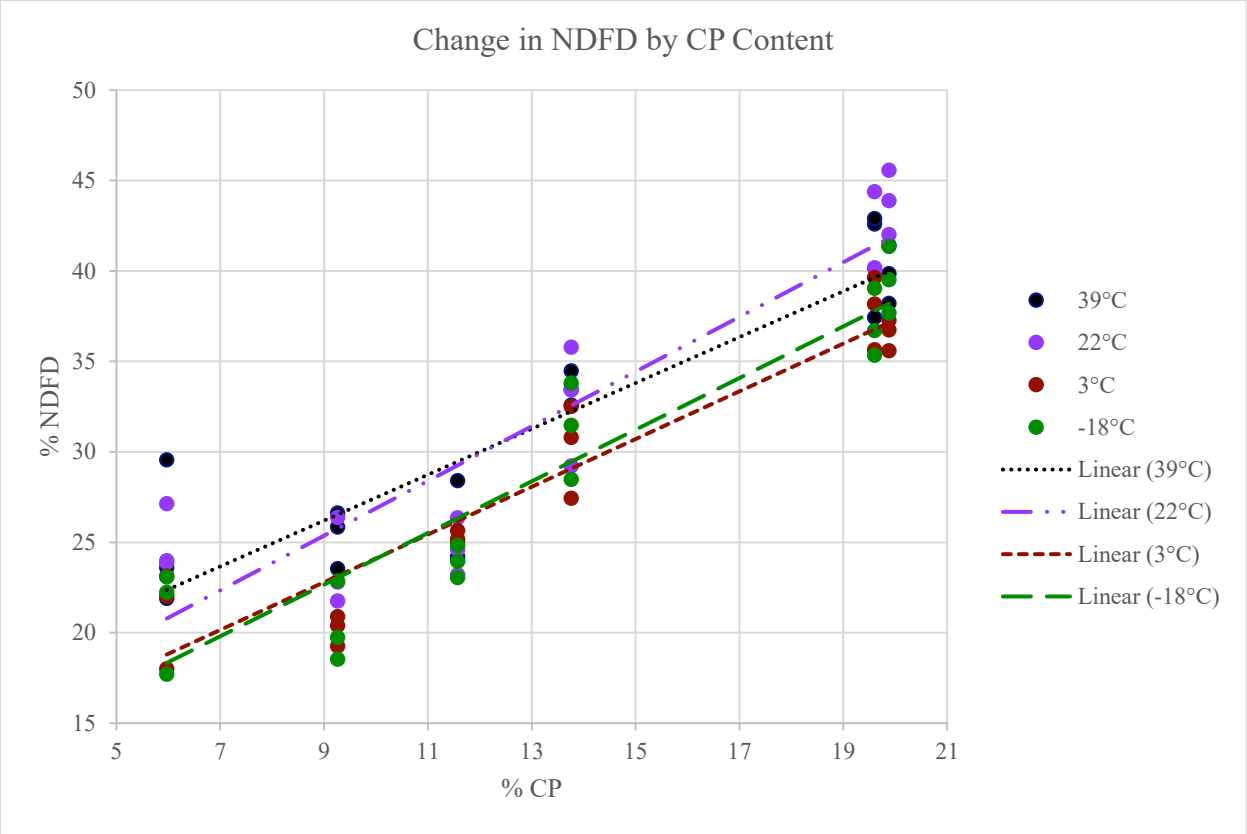


Figure 9. Microbial inoculum storage temperature by crude protein interaction in equine neutral detergent fiber digestibility of hay regression analysis of covariance.

Table 11. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine neutral detergent fiber digestibility of hay with crude protein as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.5053 | 1.0384 | 0.9618 |
| 39°C | 3°C | 3.1749 | 1.0384 | 0.0165* |
| 39°C | -18°C | 3.7422 | 1.0384 | 0.0033* |
| 22°C | 3°C | 2.6696 | 1.0384 | 0.0584* |
| 22°C | -18°C | 3.2370 | 1.0384 | 0.0140* |
| 3°C | -18°C | -0.5674 | 1.0384 | 0.9472 |

* indicates significant p-value (P<0.05).

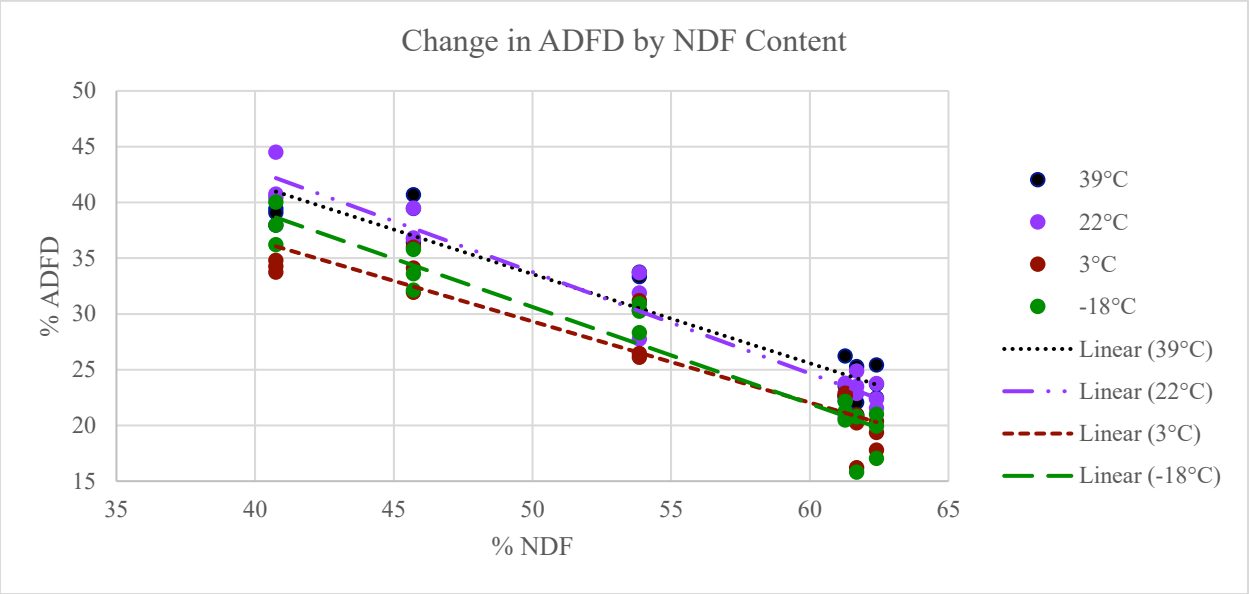


Figure 10. Microbial inoculum storage temperature by neutral detergent fiber interaction in equine acid detergent fiber digestibility of hay regression analysis of covariance.

Table 12. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine acid detergent fiber digestibility of hay with neutral detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.2800 | 0.9223 | 0.9902 |
| 39°C | 3°C | 3.2848 | 0.9223 | 0.0037* |
| 39°C | -18°C | 2.7925 | 0.9223 | 0.0179* |
| 22°C | 3°C | 3.5648 | 0.9223 | 0.0014* |
| 22°C | -18°C | 3.0725 | 0.9223 | 0.0075* |
| 3°C | -18°C | -0.4923 | 0.9223 | 0.9505 |

* indicates significant p-value (P<0.05).

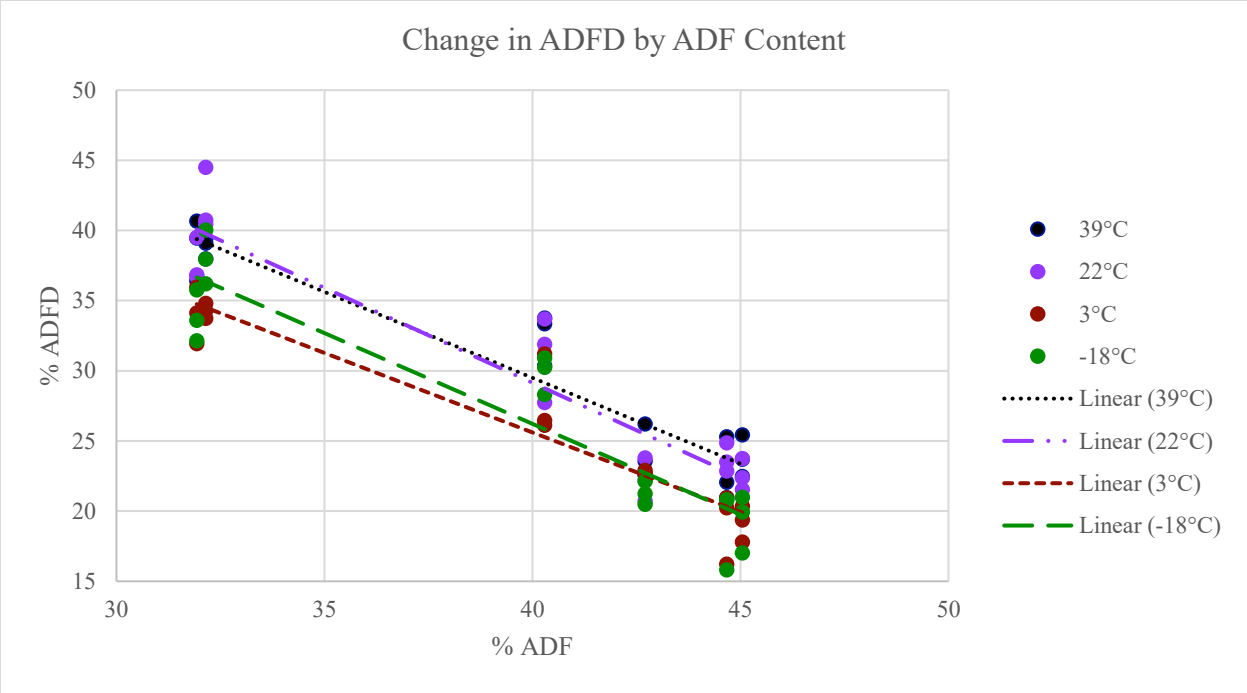


Figure 11. Microbial inoculum storage temperature by acid detergent fiber interaction in equine acid detergent fiber digestibility of hay regression analysis of covariance.

Table 13. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine acid detergent fiber digestibility of hay with acid detergent fiber as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.2800 | 0.9156 | 0.9900 |
| 39°C | 3°C | 3.2848 | 0.9156 | 0.0034* |
| 39°C | -18°C | 2.7925 | 0.9156 | 0.0169* |
| 22°C | 3°C | 3.5648 | 0.9156 | 0.0013* |
| 22°C | -18°C | 3.0725 | 0.9156 | 0.0070* |
| 3°C | -18°C | -0.4923 | 0.9156 | 0.9495 |

* indicates significant p-value (P<0.05).

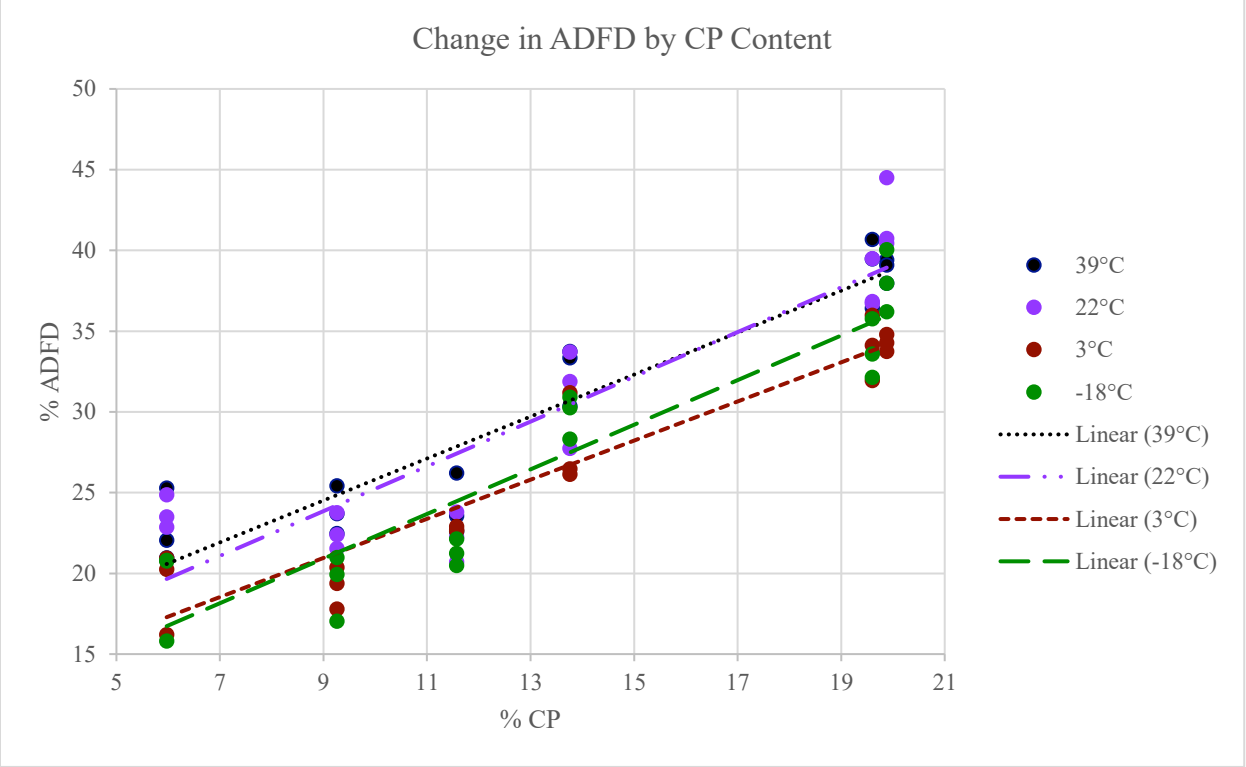


Figure 12. Microbial inoculum storage temperature by crude protein interaction in equine acid detergent fiber digestibility of hay regression analysis of covariance.

Table 14. Difference of least squares means by storage temperature of microbial inoculum for linear regression of equine acid detergent fiber digestibility of hay with crude protein as a covariate with Tukey's Honest Significant Difference test results.

| | | Tukey Pairwise Comparison Results | | |
|---|---|---|---|---|
| Pairwise Comparison | | Estimate | Standard Error | Adjusted P-Value |
| 39°C | 22°C | 0.2245 | 0.7211 | 0.9935 |
| 39°C | 3°C | 3.5626 | 0.7211 | 0.0145* |
| 39°C | -18°C | 2.8480 | 0.7211 | 0.0495* |
| 22°C | 3°C | 3.7871 | 0.7211 | 0.0067* |
| 22°C | -18°C | 3.0725 | 0.7211 | 0.0251* |
| 3°C | -18°C | -0.7145 | 0.7211 | 0.9664 |

* indicates significant p-value (P<0.05)

## DISCUSSION

Analysis of DMD, NDFD, and ADFD showed that both fresh fecal material held at 39°C that was immediately used and fecal material held at 22°C for 6h provided microbial inoculum that, when incubated with forage samples, yielded similar digestibility results. These results indicate that short term storage of fecal material for microbial inoculum is possible, and that collecting naturally deposited fecal material within 6h of deposit from the ground during 22°C weather is also possible. Both storage at refrigerator temperatures of 3°C for 6h and freezer storage at -18°C for 24h adversely affected digestibility results by reducing estimated digestibility values, suggesting that cold storage of fecal material is not a viable option for forming microbial inoculum for digestibility studies.

Exposing fecal material to oxygen for up to 6h does not significantly impact the microbial population present in the fecal material, as no difference in digestibility was seen between microbial inoculum formed from fresh fecal material and that formed from fecal material left at 22°C for 6h in an open bag. Previous research has established that the majority of bacterial species in the hindgut of the horse, and therefore in fecal material, are anaerobic organisms that are oxygen-sensitive, and it would be expected that these bacteria would not survive when placed in an anerobic environment (Harlow et al., 2015; Julliand and Grimm, 2016). Even in anerobic conditions, storage for 2h has been shown to significantly reduce cellulolytic bacteria populations in fecal material by over 90% when stored in 1g samples (Harlow et al., 2015). Fecal material in the present study was stored as whole fecal material in 45g allotments; it is possible that the larger amount of material stored allowed the inside environment of the fecal material to remain relatively anaerobic.

Inconsistent with previous research by Hervás et al. (2005) that found that chilled rumen fluid stored on ice at 0°C for either 3 or 6h was a suitable alternative to fresh rumen fluid for in vivo digestibility studies, in the present study fecal samples chilled at 3°C for 6h provided DMD estimates that were 4.13 percentage points lower than fresh samples. Consistent with prior research, in this study frozen fecal samples significantly reduced DMD estimates by an average of 4.22 percentage points. Both frozen rumen fluid from cattle and frozen fecal material from equines have been previously shown to reduce digestibility estimates when used to form microbial inoculum (Hristov et al., 1999; Murray et al., 2012). In contrast to work by Murray et al. (2012) that found the reduction in digestibility when using previously frozen inoculum was substrate dependent, with only low quality, high ADF forages being affected, the reduction in digestibility seen in this study was not substrate dependent, with the CP, NDF, and ADF values of the forage having no effect on the magnitude of the reduction in DMD, NDFD, or ADFD. Microbial populations are not equally susceptible to damage from freezing, as cellulolytic bacteria are primarily G- and are less resistant to freezing injury than G+ bacteria, likely explaining the substrate-dependent effect of frozen inoculum on digestibility in earlier studies (Miyamoto-Shinohara et al., 2000; Sutcliffe, 2010; Murray et al., 2012).

Unexpectedly, there was no difference observed between the two cold treatments of microbial inoculum, suggesting that microbial death is likely not the reason for the reduction in digestibility, as it would be expected that freezing at -18°C would result in higher death rates than storage at 3°C, and that higher death rates would result in a concurrent decrease in digestibility estimates (Miyamoto-Shinohara et al., 2000). It is possible that the reduction in DMD, NDFD, and ADFD that occurred was the result of microbial lag. Bacteria subjected to cold storage are known to have a lag time of several hours before bacteria begin multiplying and

producing enzymes at a normal rate (Ray and Speck, 1973). This lag time may explain the several percent reduction in digestibility values observed in this study in the 3°C and -18°C stored samples.

**Limitations**

This study used microbial inoculum formed from pooled fecal samples. Previous research has shown large individual variations in microbial communities and in forage digestibility between individual horses (Lattimer et al., 2007; Earing et al., 2010; Dougal et al., 2013). Pooling fecal samples in the present 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 presents a true average of digestibility results. It is possible that combining fecal samples from multiple individuals that may have different microbial species present in the hindgut results in a pooled sample with greater bacterial diversity than any one individual has, which may result in the overestimation of digestibility values if this larger diversity in the bacterial community results in more of a given forage substrate being digested (Julliand and Grimm, 2016). It is also possible that the larger bacterial diversity present in a pooled fecal sample is inconsequential due to the large amounts of functional overlap in bacterial communities (Dougal et al., 2014; Ericsson et al., 2016; Julliand and Grimm, 2016).

A wide range of temperatures was present between the tested levels of 39°C, 22°C, 3°C, and -18°C in this study. Further research will be needed to determine more specific bounds for temperature and time limits for storage of microbial inoculum between the tested storage temperatures of 22°C and 3°C.

# CONCLUSION

Fecal material collected from the ground within 6h of deposit during 22°C weather is a viable alternative for forming microbial inoculum for in vitro digestibility studies when the collection and use of fresh fecal material is not possible. Validation of the use of stored fecal material for the formation of microbial inoculum for in vitro digestibility studies provides researchers with the ability to use naturally deposited fecal material, to use inoculum from animals located further distances from laboratory facilities, to transport inoculum in a wider variety of temperatures and environmental conditions, and aids in standardizing in vitro research procedures and results. Both chilled and frozen fecal material provided digestibility estimates that were significantly lower than fresh inoculum, suggesting that cold stored fecal material should not be used to form microbial inoculum for in vitro digestibility studies. Six hours at 22°C provided viable microbial inoculum in this study; further research will be needed to determine the maximum length of time that storage is possible and to determine the effects of other storage temperatures not tested in the present study.

Contrary to previous studies, this study did not find that the reduction in digestibility estimates seen when cold stored fecal material was used to form microbial inoculum were substrate dependent. Multiple possibilities, including microbial lag time, microbial damage, and microbial death may explain the observed reduction in digestibility estimates. Further studies using larger amounts of cold stored microbial inoculum may help elucidate the role of bacterial damage and death in the decline in digestibility estimates. Trials with fecal material that has been cold stored, then thawed and warmed for several hours before being used to form microbial inoculum would provide information on whether microbial lag time is involved in the observed

reduction in digestibility values. Additionally, bacterial enumerations and next generation sequencing would provide valuable information as to the mechanisms behind the observed decline in digestibility seen with microbial inoculum from cold stored fecal material.

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