Changes in *in vitro* fermentative capacity of equine feces due to alteration of forage diet

by

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Abstract

In vitro digestibility methods require a microbial inoculum source. In hindgut fermenters like equids, cannulation of the cecum is a high-risk procedure and can be avoided by using feces as a microbial inoculum source. The hindgut microbial population can change with an alteration of concentrate and forage ratios in equids. This study's purpose was to determine if forage type fed to horses providing fecal inoculum alters subsequent *in vitro* digestibility estimates. Four mature mares were arranged in a 2 × 2 crossover design and used in a 4-wk study using two species of hay fed ad libitum: alfalfa (*Medicago sativa*) and Coastal bermudagrass (*Cynodon dactylon*). Each of two periods consisted of 7 d during which the mares were adapted to their respective diets, followed by feeding the experimental forage diet for 7 d. On d 14 of each period, feces were collected via rectal grab from each mare. Fecal samples (200 g) were blended with 400 mL of buffer solution under anaerobic conditions, filtered to remove large particulates, and placed into one of eight Daisy^{II} (ANKOM Technology) incubation vessels. Filter bags were filled with 0.25-g samples of dried, ground alfalfa and bermudagrass to pass a 2-mm screen. Six filled filter bags of a single forage plus 2 blanks were added to each vessel before being flushed with CO_2 and sealed for fermentation at 39.5°C. Half of the sample bags were removed at 48 h, and the remaining bags were removed at 72 h. Sample bags were rinsed with cold deionized water and dried at 60°C for 12 h before weighing for determination of percentage dry matter in fermentation residues. Dry matter disappearance (DMD) was calculated, followed by NDF and ADF determination. Using JMP Pro 12 (SAS Inc.), an ANOVA was performed. Significance

was declared at $P < 0.05$. Results from the 48-h fermentation indicated that donor animal diet had no effect on DM, NDF, or ADF digestibility of bermudagrass, but at 72 h, DM digestibility estimates are higher with alfalfa-sourced feces. For alfalfa at 48 and 72 h, DMD, NDF, and ADF digestion were more extensive when fermented in alfalfa-sourced feces than bermudagrass-sourced feces. These results indicate that equids used as microbial inoculum donors for *in vitro* digestibility evaluations may need to be consuming the forage being studied, but further investigation is required.

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I. REVIEW OF LITERATURE

Fermentation and Microbial Inoculum

Digestibility estimations made *in vivo* can be invaluable for determining the extent to which a ruminant or non-ruminant herbivore can digest a feedstuff. However, *in vivo* measures have limitations and thus considerations must be made to identify alternative methods. *In vivo* digestibility estimations can be costly to perform, and require that the animal's mobility be somehow restricted for total fecal collection (Sales, 2012).

In vitro fermentations can be performed in place of *in vivo* fermentations. A fermentation technique developed by Tilley and Terry (1963) is a method for performing digestibility estimates with small samples of feedstuffs requiring no lengthy *in vivo* measurements. In this technique, a sample of microbial cells from a ruminant animal's rumen is required to create an inoculum with which to ferment feedstuffs. The researchers subjected feedstuffs to pepsin digestion after the *in vitro* fermentation with rumen liquor to mimic the digestion that occurs within the ruminant digestive tract, and concluded that this technique was a viable substitute for *in vivo* measurements.

A procedure similar to that developed by Tilley and Terry (1963) was performed by Applegate and Hershberger (1969). In this experiment, cecal microflora were collected from equids and used to perform *in vitro* fermentation. Researchers found that the technique originally developed with ruminant animals in mind is also applicable to non-ruminant herbivores such as equids.

However, it is important to note that because equids are hindgut fermenters, these animals subject ingesta to pepsin digestion before microbial fermentation can occur. Digesta that exits the cecum only passes through the large intestine where microbial fermentation can

continue (Lowman et al., 1999). Very little, if any, enzymatic digestion of microbial cells occurs in the equine hindgut.

To mimic this, Abdouli and Attia (2007) performed an enzymatic pre-digestion on various feedstuffs including oat hay, barley grain, and soybean meal to establish an *in vitro* method that accounts for both pre-cecal and post-ileal digestion. These samples were subjected to porcine pepsin followed by porcine amylase. Afterwards, samples were incubated in fecal microbial inoculum to mimic the sequence of digestion that occurs in the equine digestive tract. These researchers concluded that with high-fiber feeds such as the oat hay, only the *in vitro* fermentation procedure is needed. The enzymatic pre-digestion was only necessary with low-fiber samples such as the barley grain and soybean meal. In fact, enzymatic pre-digestion was found to delay the onset of fermentation (Abdouli and Attia, 2007).

Microflora

The anaerobic microflora population of ruminant and nonruminant herbivores plays a critical role in the breakdown of consumed forage, as mammalian animals do not possess the enzymes necessary to digest fibrous plant material. Microorganisms present within the equine digestive track perform anaerobic fermentation on consumed plant material, producing VFA that the animal can absorb and use for energy. Many factors can affect the makeup of the microbiome, including but not limited to diet, health, season, and normal inter- and intra-horse variation (Coasta and Weese, 2012; Respondek et al., 2008; Berg et al., 2005). Because of the wide variety of factors affecting the microbiome makeup, it is suggested that horses intended to be microbial inoculum donors should be subjected to uniform management practices (Costa and Weese, 2012).

While microorganisms colonize the entirety of the equine digestive tract, the population located within the hindgut is more diverse than that located in the foregut, as is expected when the important role of the hindgut in microbial fermentation is considered (Costa et al., 2015). As much as 92% of the microbiome of the equine hindgut is composed of cellulolytic bacteria, and as many at 72% of these may belong to the phylum Firmicutes (Costa and Weese, 2012; Daly et al., 2001; Costa et al., 2015). Because the animal's natural diet consists mainly of forages, this large proportion of fibrolytic bacteria comes as no surprise.

Inoculum Source

The microbial content of equine feces has been found to primarily contain the phylum Firmicutes, much like the cecum. This fecal microbiome is similar to the cecum and large colon on the class and higher taxonomic levels. Feces can be used as a representation of the equid hindgut (Costa et al., 2015).

Lowman et al. (1999) performed *in vitro* fermentations with equine cecal and fecal inoculum in gas production techniques. While these researchers concluded that equine feces or cecal fluid can be used to form viable microbial inoculum, the researchers cautioned that a different prediction equation should be used for each when estimations of *in vivo* digestibilities are the goal. It has been shown that, while fecal and cecal inocula yield results that are different from one another, they are not different from *in vivo* estimations (Lowman et al., 1999; Earing et al., 2010).

The microbial fermentative activity of feces is affected by the time of collection relative to the feeding schedule of the donor animal. Fecal samples contain the greatest amount of viable and active microbial cells when collected 2 h after a morning meal (Desrousseaux et al., 2012). These fecal samples should not be preserved by freezing. This limits the microbial activity and

hampers any *in vitro* fermentation, so fecal samples should be used quickly after collection (Murray et al., 2012).

Furthermore, while the equine cecum can be surgically altered to allow access to cecal fluid for the purposes of obtaining microbial inoculum, cannulation can be dangerous to the animal's health. Cannulation can impact a hindgut fermenter's quality of life and longevity due to complications of the procedure, and should be avoided if possible. Additionally, research has indicated that cannulation may alter digestion within the animal. Pulse et al. (1973) examined the digestive ability of horses *in vivo* after cannulation and found that crude fiber digestion significantly increased, but DM digestibility estimations were unchanged. Based upon the retention time of dietary markers, the researchers concluded that, due to the cannulation of the equid hindgut, retention time of digesta had increased, allowing the microbial population a greater period of time during which to digest fibrous material (Pulse et al., 1973). Thus, surgical alteration of equids should be avoided from an animal welfare, cost, and result accuracy standpoint.

Batch Culture Fermentation

The Daisy^{II} Incubator (ANKOM Technology) has been used with equine fecal inoculum to perform *in vitro* batch culture fermentations with results compared to *in vivo* digestibility estimations. In a study performed by Earing at al. (2010), diet samples were fermented for periods of 24, 48, and 72 h, and these fermentation results were compared with those found *in vivo* by measuring NDF and DM digestibility. As fermentation period increased, the *in vitro* DM digestibility estimations grew closer to *in vivo* estimations, with 72 h of *in vitro* fermentation not differing from *in vivo* measures. In this study, the 30-h fecal inoculum fermentation DM

digestibility results obtained by Earing et al. (2010) were similar to those found by Applegate and Hershberger (1969) using cecal inoculum at 24 h of fermentation.

Similarly, Lattimer et al. (2007) collected equine fecal inoculum for *in vitro* fermentation in the Daisy^{II} Incubator. These researchers compared *in vitro* results to their own *in vivo* observations and found no differences. The convenience of the Daisy^{II} was noted by the researchers in that it allows for the fermentation of multiple samples simultaneously, has a high repeatability, and constantly mixes the contents of each individual fermentation vessel. The Daisy^{II} can be utilized to accurately predict the DM digestibility of total mixed diets that contain various ratios of concentrate and roughage (Lattimer et al., 2007).

Lattimer et al. (2007) concluded that a 48-h fermentation period was sufficient to provide *in vitro* results not different from *in vivo*, contrasting with the conclusion that a 72-h fermentation period was required by Earing et al. (2010). This discrepancy in fermentation period required to reach *in vivo* estimations was acknowledged by Earing et al. (2010), noting the differences in NDF concentration of the diets as a possible cause. Lattimer et al. (2007) used a high-fiber diet consisting of alfalfa that contained 33.14% NDF, whereas the high and low-fiber alfalfa and alfalfa-oat diets used by Earing et al. (2010) contained 51.6% and 44% NDF respectively. This difference in NDF concentration of the diets led to the conclusion that diets with high fiber forages may require *in vitro* incubation periods longer than 48 h in order to reliably estimate *in vivo* digestibility values (Earing et al., 2010).

Sample Size

The nature of the samples themselves that are fermented in a Daisy^{II} Incubator can alter the results obtained. This variation occurs as a result of both the weight and individual sample particle size. Samples weighing both 0.25 g and 0.50 g have been fermented. After a 48-h

incubation period, samples weighing 0.25 g were more extensively digested compared to 0.50 g samples when evaluated for DM, NDF, and ADF digestibility. These results suggest that a small sample size incubated for 48 h yields *in vitro* results that are not different from *in vivo* (Lattimer et al., 2007). Samples ground to pass through a 2-mm Wiley Mill screen and incubated 72 h were not different from *in vivo* results (Earing et al., 2010). These results indicate that there is a relationship between the size of the sample particles and the ratio of sample to fluid within each incubation vessel, influencing the accuracy of the *in vitro* results.

Donor Diet and Its Effects on:

Microbiome

It has been well-established that the diet of an animal, ruminant or non-ruminant, influences the makeup of the microbial population within its digestive tract. The microflora population in the rumen and equine hindgut adapt specifically to dietary changes. These changes can include changes in concentrate:forage ratios, transitioning between fresh and stored forages, or supplementation with exogenous enzymes (Medina et al., 2002; Fernandes et al., 2014; Murray et al., 2005). Willing et al. (2009) examined the microbiome in the hindgut of six horses to determine the effects of high-forage vs. high-carbohydrate diets on the population makeup and found that the proportion of the phylum Firmicutes can range from 46% to 73% of the total microbiome. These researchers also found that the stability of the microbiome's makeup was greater in horses consuming high-forage diets and did not fluctuate as greatly as those consuming concentrate. Similar results were obtained by Daly et al. (2011), who noted that, in addition to high-forage diets promoting greater proportions of phylum Fibrobacter, diets including concentrate encourage the family Clostridiaceae and phylum Bacteroidetes microbes to increase in prevalence.

In the case of fructooligosaccharide supplementation, significant changes have been observed in the populations of lactobacilli, streptococci, and total anaerobes in the equid hindgut microflora (Respondek et al., 2008).

Feeding grain to equids can cause non-structural carbohydrates (NSC) such as starch to reach the hindgut when fed in amounts above 0.4% BW (Potter et al., 1992). This can cause the large intestinal microbial content to achieve greater overall concentrations of bacteria but lower proportions of cellulolytic bacteria than equids fed an all-forage diet (Julliand et al., 2001). Additionally, a diet high in NSC can increase the concentration of bacteria in the hindgut that produce lactate, prompting the researchers to conclude that both the microbial profile and the biochemical composition of the colon are altered by the diet the animal is consuming. Related studies found that starch that escapes the equine foregut passes quickly through the cecum, and therefore has a greater impact on the colonic microbial population than that of the cecum (Drogoul et al., 2001; de Fombelle et al., 2001). In these cases, the microflora of the equine hindgut can be modified by grain and, by extension, NSC that reach the hindgut. While the total concentrations of anaerobic (microorganisms that require an environment devoid of oxygen) or cellulolytic bacteria were not observed to change in the study by de Fombelle et al. (2001), the concentration of aero-anaerobic bacteria (microorganisms that can survive in environments with or without oxygen) rapidly decreased within 5 h of a change in diet.

Conversely, Moore and Dehority (1993) and Murray et al. (2014) found results that indicate neither the cellulolytic nor amylolytic bacteria concentration within the equine hindgut were altered by high-fiber or concentrate diets, nor was there an effect on cecal protozoa concentrations. However, an increase in concentration of colonic protozoa was noted when animals were fed the high-concentrate diet. Overall, microbial concentrations were unchanged in

the cecum, but were increased in the colon when concentrate was added to the diet (Moore and Dehority, 1993).

Higher levels of concentrate in the diet have been found to allow Gram-positive bacteria to proliferate, producing lactic acid, at the expense of Gram-negative bacteria, those responsible for fiber degradation (Murray et al., 2014). Despite this shift from Gram-negative toward Gram-positive bacteria, the counts of cellulolytic and amylolytic bacteria within the feces showed no differences between horses fed low or high-concentrate diets. The researchers proposed that this increase in Gram-positive bacteria was a consequence of greater substrate concentrations present within the colon.

Changes in the microbiome of the equine hindgut can occur rapidly and can be detected in fecal material within 4 d of the animals being transitioned from an ensiled forage-grain diet to pasture (Fernandes et al., 2014). de Fombelle et al. (2001) noted a rapid decrease in the hindgut aero-anaerobic bacteria within 5 h of an abrupt dietary change, and Medina et al. (2002) noted that both microflora profile and activity were altered by changing the NDF:starch ratio of the diet within 1 h. When NSC are delivered to the hindgut by either rapid increase in diet or by overwhelming the foregut's capacity for digestion, the ventral colon is the main location of microbial profile upset (Drogoul et al., 2001).

The form of the forage within the diet may also affect the microbial population. Forage processing, such as by chopping or cubing, alters the way in which the animal consumes it, and mean particle size present within the digestive tract changes. Mean retention time and overall rate of passage of digesta through the compartments of the digestive tract may be changed, possibly altering the fiber digestibility, the relative proportion of microbes found within each compartment, and daily fecal output of the horse (Potts et al., 2010).

The health status of the horse should also be considered when selecting animals from which to collect microbial inoculum. Equids afflicted with equine metabolic syndrome (EMS) have decreased diversity of fecal microbes compared with metabolically normal horses consuming the same hay-based diet. This decrease in diversity is accompanied by a decrease in the proportion of the genus *Fibrobacter*. As a consequence of this decreased microbial diversity, the ability of the microbiome to adjust and respond to changes of diet is likely diminished (Elzinga et al., 2016). Fecal inoculum from horses prone to laminitis were also compared with normal horses consuming the same diet. In this study, even though it has been hypothesized that the microbial population between these groups of horses differs, the fermentative capacity of the feces was not different with the exception of pH. It is important to note, however, that Murray et al. (2009) utilized frozen feces as an inoculum source due to necessity and that may have altered or biased results (Murray et al., 2012).

The extent to which these microbiome population shifts affect *in vitro* digestibility estimates has been investigated using various measurement methods and multiple feedstuffs including grains, concentrates, and forages.

Grain and Concentrate

Diets that incorporate various grain:forage and concentrate:forage ratios have been studied. Earing et al. (2010) investigated four diets and their effect on subsequent *in vitro* digestibility estimates: alfalfa hay, alfalfa hay with oats, timothy hay, and timothy hay with oats. Using fecal inoculum from horses consuming the respective forage, the inclusion of oats in the diet was observed to cause no differences in *in vitro* DM or NDF digestibility. It is likely due to oats being largely digested within the foregut of the animal, and the majority of the oats do not reach the hindgut.

Rumen fluid from cattle and sheep consuming several forage:concentrate ratios was used in an *in vitro* gas production technique. It was found that donor diet affects neither NDF degradation nor total gas production, but causes different estimations of organic matter (OM) digestion, ADF degradation, VFA production, and the acetate:propionate ratio. These findings led to the conclusion that, while there is limited adaptation of the microbial population to conditions within the *in vitro* system, donor diet affects certain *in vitro* estimates, and donor animals may require adaptation to the diet being evaluated before the inoculum sources are collected to yield the most accurate and biologically relevant results (Boguhn et al., 2013).

At a constant energy intake, increasing the amount of concentrate in a diet will increase OM digestibility and decrease that of NDF and ADF. In conclusion, as concentrate increases as a proportion of the diet, fiber digestibility decreases (Drogoul et al., 2001). In studies of similar design where equids were fed low and high-concentrate diets, the fecal parameters were determined to be similar. Murray et al. (2014) found no difference in VFA production or lactate present in the feces, leading the researchers to the conclusion that the effect of low vs high-starch diets on equine fecal parameters is minimal. Murray et al. (2006) also found that diet did not affect total gas volume or DM degradation, but may affect observed lag times in *in vitro* fermentations and thus concluded that microbial inoculum should be collected from multiple animals. Furthermore, those animals should be consuming feedstuffs that are similar to those being tested.

Earing et al. (2010) examined the effects of adding oats to alfalfa or oats to timothy hay diets. This addition of oats was found to make *in vivo* NDF digestibility more difficult to accurately predict. However, estimations made *in vitro* showed that the presence of oats within the diet of the inoculum donor animal had no effect on DM or NDF digestibility. Furthermore,

the researchers noted that, when multiple substrates are incubated within the same vessel, one substrate may alter the fermentation of another by causing the microbial population within the vessel to be altered (Earing et al., 2010).

The apparent DM digestibility of the equine diet was not improved by supplementing the diet with concentrate. In fact, the increased digestibility of NSC within the small intestine in the high-concentrate diet was counteracted by an accompanying decrease of fiber digestion within the hindgut (Holland et al., 1998).

Different levels of starch in the diet can affect DM losses seen in *in vitro* fermentation but not gas production when equine fecal inoculum is utilized (Murray et al., 2003).

Forage

Similar to grains, the extent to which the effect of forage on the equine hindgut microbiome affects *in vitro* fermentation estimates is contested. Studies on the effect of inclusion of a low-fiber feedstuff in the diet performed by Murray et al. (2003) and Boguhn et al. (2013) found that the roughage source likewise caused changes in the fermentative capacity of cattle rumen and equine fecal inoculum. These changes are not uniform, however, and only affected gas production, OM digestibility, and ADF degradation.

The type of roughage in the diet affected the degradation of ADF when rumen fluid was used as inoculum (Boguhn et al., 2013). In the first trial of this study, cannulated cattle and sheep were fed diets consisting of either grass or corn silage, and rumen fluid was collected as a source of microbial inoculum. Diet samples were fermented with rumen fluid and evaluated for CP, OM, NDF, and ADF degradation as well as VFA production. Boguhn et al. (2013) found that the effect of the all-silage diets caused a significant difference in *in vitro* OM and ADF degradation and in the acetate:propionate VFA ratio, but donor animal diet caused no change in CP or NDF

degradation. Thus, the researchers concluded that ruminant animals should be adapted to the diet that is to be subjected to *in vitro* fermentation.

Future Research and Implications

Extensive research has been performed evaluating the effect a low-fiber feedstuff addition to the animal diet has on *in vitro* digestibility values, but there is little conclusive research on dietary effects on donor inoculum fermentation parameters. *In vitro* fermentations are relatively easy to perform, allow for many replications, and require fewer resources and time than *in vivo* evaluations.

It is unclear if such changes in the microbial population significantly alter *in vitro* digestibility estimates in horses. If donor diet has a significant effect on IVDMD, the horse should be adapted to the forage of interest before collecting feces for use in *in vitro* fermentation.

If no effect is found, as suggested by Applegate and Hershberger (1969), IVDMD can be accurately performed on novel feedstuffs with equine feces collected from any donor animal without adapting the animal to a novel diet, which would be especially significant when there is a limited amount of forage available for testing.

II. CHANGES IN *IN VITRO* FERMENTATIVE CAPACITY OF EQUINE FECES DUE TO ALTERATION OF FORAGE DIET

INTRODUCTION

With new cultivars of forages for livestock continually in development, it is imperative that researchers are able to produce reliable and biologically relevant digestibility estimates for the livestock that will be consuming it. *In vivo* research can provide digestibility estimates that are accurate, but these procedures require financial and animal resources that are not always available. As an alternative, *in vitro* work can be performed with a source of microbial inoculum from a donor animal, and allows fermentation to be carried out that is similar to that occuring *in vivo* but does not require large amounts of animal input. Thus, this type of *in vitro* fermentation often requires a smaller overall input of resources than *in vivo* experimentation. Additionally, many different samples may be examined during the same period of time as an *in vivo* study. *In vitro* digestibility measures cannot completely replace *in vivo* measures, but they provide invaluable estimations of the potential extent a feedstuff can be digested within the live animal.

Where ruminant animals are concerned, ruminal cannulas can be used to obtain source inoculum. Cannulation generally does not impact a ruminant animal's quality of life or longevity, and it provides relatively easy access to ruminal fluid for microbial inoculum to be used in *in vitro* fermentations. Hindgut fermenters such as equids perform a large proportion of microbial fermentation within the cecum, but cannulation of this structure is hazardous to the animal. The surgical procedure itself is more dangerous than ruminal cannulation, and equids face an increased risk of peritonitis and other serious and life-threatening complications even after the cannula is in place. Fortunately, fecal matter can serve as a safe, biologically suitable substitute for microbial inoculum in the case of hindgut fermenters to yield DM, NDF, and ADF

digestibility estimations. Shifts in microbiome population makeup must occur to adapt to the diet that the horse is consuming.

In vitro fermentations performed with microbial inoculum provide estimates that are not different from *in vivo* measures, but the effect of a strictly forage-based diet on *in vitro* digestibility estimates is still unknown. To investigate the extent to which diet alteration affects the population shift and its subsequent effect on *in vitro* measures, two pairs of horses were fed one of two diets in a crossover design. *In vitro* fermentation was carried out in this study, and measures were compared between horses consuming the diet being evaluated and a novel hay sample. This objective of this study was to determine if any significant differences were caused in DM, NDF, or ADF digestibility estimations based on inoculum donor diet. If a difference exists, the secondary objective was to investigate if it could be overcome by extending the fermentation period from 48 to 72 hr. If no differences exist, these results could prove beneficial for future research, as fecal inoculum could be collected from a horse and used to evaluate a variety of novel feedstuffs without having to take donor diet into account or adapt an equid to a novel diet in order to obtain relevant digestibility estimates.

MATERIALS AND METHODS

Horses

Three American Quarter Horse mares and one Arabian × American Quarter Horse cross mare with a mean age of 12.75 ± 4.92 yr were used in this 2 × 2 crossover design experiment. The mean weight was 523.9 ± 34.49 kg at the beginning of the study, with a mean body condition score (BCS) of 6.1 ± 0.72 (Henneke et al., 1983). All live animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Mares were maintained in pairs at the Auburn University College of Veterinary Medicine Equine Reproduction Center in outdoor pens with shelter and hay feeders. Each pen allowed the mares access to automatic waterers and trace mineralized salt blocks (Appendix 1) for *ad libitum* intake. The pens had minimal amounts of fresh, growing forage.

Feeding

The project was divided into two 2-wk feeding periods. Mares were paired and assigned 1 of 2 dietary treatments, receiving either bermudagrass (*Cynodon dactylon*, var. 'Coastal') or alfalfa (*Medicago sativa*) hays beginning at rates of 2.5% BW daily and increasing until *ad libitum* intake was reached. The bermudagrass and alfalfa hays were first-cutting sourced from commercial producers from Reeltown, AL (32.3613º N 85.4819º W) and Danville, AL (34.4145º N, 87.0875º W) respectively, and were stored to prevent exposure to rain and excessive sunlight.

Each feeding period began with a 7-d transition period during which mares were acclimated to the assigned diet. Following the transition period was a 7-d period during which mares consumed only the experimental diet. Mares were fed once daily at 0700 h.

Forage Sample Preparation

Sixteen bale-core samples were taken from each hay type and combined for subsequent grinding by a Model 4 Thomas-Wiley Laboratory Mill through a 2-mm screen. These aggregates served as a representative sample of each hay type and were used for all laboratory analyses. ANKOM filter bags were washed with acetone, dried, labeled, and weighed. Samples of ground hay weighing 0.25 g were sealed into the bags. Twenty-four filter bags were filled with bermudagrass hay, and 24 were filled with alfalfa hay. An additional 16 empty bags were sealed to serve as blanks for the calculation of a correction factor. Additional bags were filled with 0.25 g of sample, sealed, and set aside for DM, NDF, and ADF determinations.

Fecal Sample Collection

Fecal collections were performed on d 14 and 28 of the study. Collections were performed beginning at 0900 h, 2 h after the morning meal, to maximize the number of viable and active microbial bodies within the feces (Desrousseaux et al., 2012). Feces were collected via rectal grab from all four mares by the same researcher, and were immediately placed into resealable plastic bags to remain as anaerobic as possible. An effort was made to collect a minimum of 600 g of feces to ensure suitable amounts for microbial inoculum preparation. Collection time was recorded, and excess air was squeezed from the bags before being sealed and placed into a polystyrene foam container. To maintain the temperature of the feces during transportation to the laboratory, bottles were filled with 38ºC water and placed in the container alongside the fecal samples. Fecal samples were transported to the laboratory and used as inocula sources within 1 h.

Batch Culture Procedure

Inocula were prepared in the same order in which they were collected so that the effect of time spent in the resealable bags was minimized. Two hundred g of feces were placed into a blender carafe with 400 ml of buffer solution. The carafe was flushed with CO_2 for 30 seconds, sealed, and blended at the highest speed for 30 s. This blended mixture was filtered through cheesecloth to remove large particulates so that only the microbial inoculum remained.

Inoculum was immediately dispensed into a Daisy^{II} incubation vessel and continually flushed with CO_2 as the pH was adjusted to 7.0 to avoid exposure of the microorganisms to oxygen. Six filled filter bags of one forage type plus two blanks were added, and the jar was then flushed with CO_2 for an additional 30 s before being sealed and finally placed into an incubator. The time that each jar was placed into a Daisy^{II} Incubator was recorded so that fermentation of samples could be precisely terminated. At 48 and 72 h of fermentation, jars were removed from the incubators one at a time. Three filled filter bags and one blank were randomly removed from each jar for each incubation time evaluated. The jars were flushed with CO_2 for 30 s before they were replaced in the incubator following the 48-h incubation. The filter bags were rinsed with cold, deionized water until it ran clear and no visible microbial colonies remained on the exterior of the bags. Bag identification numbers were documented to record the length of fermentation. The filter bags were placed onto metal pans for drying. This process repeated for all 8 jars as they reached the final termination point of fermentation at 48 or 72 h.

Lab Analysis

After fermentation, bags were rinsed with deionized water and placed into a 60ºC oven and dried for 12 to 24 h. Samples were removed from the oven and cooled in desiccators for 15 min before weighing for DM content. Neutral detergent fiber analysis was performed, followed

sequentially by ADF analysis by the ANKOM^{2000} Automated Fiber Analyzer (ANKOM Technology). Hay samples were analyzed for crude protein (CP) via the Kjeldahl method (FOSS), ash, and total nonstructural carbohydrate (TNC) content as a measure of diet content. Equations used for DM, NDF, and ADF digestibility estimations are listed in Appendix 2.

Total nonstructural carbohydrate analysis was performed on hay samples that were ground through a 1-mm screen. Samples were evaluated in duplicate. Hay samples weighing approximately 0.50 g were placed into 600 mL beakers, and 50 mL of 0.05 N H_2SO_4 were added. Samples were boiled for 1 h, then beakers were placed within an ice bath. Once cool, pH was adjusted to between 4.4 and 4.6 by the addition of NaOH and H_2SO_4 dropwise. One mL of a 1:9 ratio of G-ZYME 480 (25% α amylase and 75% glucoamylase, DuPont) to deionized water was added, and samples were incubated at 60ºC for 1 h.

After incubation, samples were filtered using a stainless steel filtration apparatus and Watman #1 filter paper into 250 mL volumetric flasks. Two mL of 1 N NaOH were added to each volumetric flask, and the liquid was brought to volume using deionized water. The contents were mixed, and 10 mL aliquots were placed into glass test tubes. Each test tube received 10 mL of Shaffer-Somogyi reagent (AOAC, 1995) and covered with glass condensers. Samples were boiled for 15 min, and subsequently placed within an ice bath.

After cooling, 2 mL of 1:1 potassium iodide:potassium oxalate solution, 5 mL of 1 N H_2SO_4, and a stir bar were added. Samples were allowed to rest before a second addition of 5 mL of 1 N H_2SO_4 was added to each test tube.

A single drop of FastBreak® (WinField United, St. Paul, MN) was added to minimize foaming during titration, and 2 mL of a 1% starch solution was added. Titration was performed using 0.02 N sodium thiosulfate until the color of the sample changed from a dark purple to a

bright, translucent blue. The amount of titration fluid required to titrate each sample was compared to a standard curve equation determined for the Shaffer-Somogyi solution. Calculation of % TNC was performed using the sequence of equations shown in Appendix 3.

Statistical Analysis

Statistical analysis was performed using JMP Pro 12 (SAS Inc.) by oneway ANOVA. For this, DM, NDF, and ADF were response variables, and donor animal diet was the main effect. Additionally, blocks were defined as being one of the two feeding periods. Significance was declared at $P < 0.05$, and trends were noted at $P < 0.10$.

RESULTS AND DISCUSSION

Throughout the course of the study, the mares experienced no significant change in BW (mean 519.94 ± 47.22 kg) or BCS (mean 5.81 ± 1.03). Hays were initially provided at a rate of 2.5% BW (DM basis). However, alfalfa hay was consumed at a greater rate than bermudagrass. This high rate of alfalfa hay consumption was likely due to the alfalfa having a lower NDF content (46.79%) on a DM basis than bermudagrass hay (75.03%), increasing its rate of passage (Rodiek and Jones, 2012).

Because of this, the daily alfalfa allotment was divided into two equal portions and offered twice daily to maintain digestive tract health by increasing the amount of time the mares spent eating. At the same time, the daily ration of alfalfa hay was gradually increased until *ad libitum* intake was reached. All mares accepted each hay type, and no refusals occurred through the duration of the study.

During the first fecal collection on d 14 of the study, one of the mares had insufficient fecal material for microbial inoculum formation. Fecal samples from the three remaining mares were collected and transported to the laboratory for formation of inoculum and initiation of fermentation. Feces were collected from the remaining mare later the same day. This proved to have an no effect on the fermentative capacity of the fecal microbial bodies despite the fecal collection occurring more than 2 h after the morning meal.

Forage Analysis

Chemical composition of both hay types was determined (Table 1). These are indicative of average values for alfalfa and Coastal bermudagrass hays (NRC, 2007; NRC, 2016). It is important to note that legumes such as alfalfa utilize pectins as a storage carbohydrate more so than grasses such as bermudagrass. This pectin fraction is removed by neutral-detergent along

with other non-cell wall constituents, and so is not readily detectible by any of the analyses performed in the current study (Van Soest, 1982).

Table 1. Chemical composition of alfalfa and bermudagrass hays

		DM basis				
Forage Type	% DM	% NDF	% ADF	% Ash	% TNC[†]	% CP
Alfalfa	89.01	46.79	34.47	8.74	8.32	18.67
Bermudagrass	91.37	75.03	32.68	4.86	12.51	7.72

[†]Total nonstructural carbohydrate (TNC)

Alfalfa Hay Fermentation

Disappearance of alfalfa DM, NDF, and ADF was greater at both 48- (Table 2) and 72-h (Table 3) fermentation times when hay was fermented with inoculum from mares consuming alfalfa. At comparable stages of maturity, legumes such as alfalfa typically contain a greater proportion of lignin within the cell walls than grasses such as bermudagrass. However, despite this increased cell wall lignification, alfalfa total plant DM is a more readably digestible feedstuff compared with bermudagrass. This high digestibility of alfalfa despite its high lignification is due to its relatively low NDF content.

In the case of alfalfa hay samples, the effect of the inoculum source was not overcome by continued fermentation. All 72-h *in vitro* fermentation estimations (DM, NDF, and ADF) of alfalfa hay samples were greater when incubated with the alfalfa-derived fecal inoculum than with bermudagrass-derived inoculum.

For DM digestibility, an effect of period was seen at 48 h but not at 72 h of fermentation.

Period was significant for both NDF and ADF digestibility estimations of alfalfa hay at both fermentation times.

Table 2. Digestibility estimates of alfalfa hay at 48 h of fermentation using fecal inoculum from horses fed alfalfa or bermudagrass hay, expressed as mean % loss

		DM basis	
Inoculum	DM	NDF	ADF
Alfalfa	46.61[a]	25.24[a]	17.70[a]
Bermudagrass	42.66[b]	15.23[b]	10.41[b]
SE[1]	±0.937	±2.621	±1.449
P-value[2]	0.0070	0.0133	0.0019

[1]SE for the column
[2]*P*-value for the column
[a,b] Within a column, means with different superscripts are different ($P < 0.05$)

Table 3. Digestibility estimates of alfalfa hay at 72 h of fermentation using fecal inoculum from horses fed alfalfa or bermudagrass hay, expressed as mean % loss

		DM basis	
Inoculum	DM	NDF	ADF
Alfalfa	50.94[a]	30.07[a]	25.96[a]
Bermudagrass	48.32[b]	25.51[b]	20.79[b]
SE[1]	±0.757	±1.236	±1.441
P-value[2]	0.0232	0.0164	0.0191

[1]SE for the column
[2]*P*-value for the column
[a,b] Within a column, means with different superscripts are different ($P < 0.05$)

Bermudagrass Hay Fermentation

Digestibility of all bermudagrass DM, NDF, and ADF were not different at the 48- (Table 4) fermentation time when hay was fermented with bermudagrass or alfalfa-derived microbial inoculum. However, NDF digestibility of bermudagrass hay tended ($P = 0.0804$) to be greater

with bermudagrass-derived fecal inoculum. This trend in NDF digestibility did not continue to 72 h of fermentation.

By 72 h of fermentation (Table 5), however, the *in vitro* bermudagrass hay DM digestibility with alfalfa-sourced fecal inoculum was greater than with bermudagrass-sourced fecal inoculum. The percent of NDF loss of bermudagrass hay tended ($P = 0.0544$) to be greater with alfalfa-sourced fecal inoculum. This finding could suggest that the alfalfa-sourced fecal inoculum tends to be capable of digesting a substrate's DM to a greater extent than microbes accustomed to the bermudagrass.

Dry matter digestibility of bermudagrass hay was not affected by period at 48-h fermentation times, but was affected at 72 h. Digestibility of NDF was not affected by period at either 48 or 72 h. Period had no effect on ADF digestibility at 48 h of fermentation, but had an affect after 72 h.

Table 4. Digestibility estimates of bermudagrass hay at 48 h of fermentation using fecal inoculum from horses fed alfalfa or bermudagrass hay, expressed as mean % loss

		DM basis	
Inoculum	DM	NDF	ADF
Alfalfa	26.63	15.81	7.59
Bermudagrass	28.71	18.52	9.74
SE[1]	±0.871	±1.044	±1.093
P-value[2]	0.1053	0.0804	0.1798

[1]SE for the column
[2]*P*-value for the column

Table 5. Digestibility estimates of bermudagrass hay at 72 h of fermentation using fecal inoculum from horses fed alfalfa or bermudagrass hay, expressed as mean % loss

		DM basis	
Inoculum	DM	NDF	ADF
Alfalfa	32.72[a]	23.61	13.55
Bermudagrass	29.41[b]	20.04	11.07
SE[1]	±1.093	±1.241	±1.277
P-value[2]	0.0441	0.0544	0.1835

[1]SE for the column
[2]*P*-value for the column
[a,b] Within a column, means with different superscripts are different ($P < 0.05$)

Discussion

The results for alfalfa hay *in vitro* digestibility are in agreement with Boguhn et al. (2013) regarding the effect of forage diet on *in vitro* ADF digestibility estimations, but contrast in regards to NDF degradation. In the current study, DM, NDF, and ADF *in vitro* digestibility estimations of alfalfa hay and DM *in vitro* digestibility of bermudagrass hay were affected by donor animal diet. Boguhn et al. (2013) fed a grass silage or a corn silage diet to animals. The difference between the NDF content on a DM basis of the grass silage (39.8%) and corn silage (37.5%) used by Boguhn et al. (2013) was 2.3% NDF and was not as pronounced as that in the current study with alfalfa (46.79%) and bermudagrass hays (75.39%) with a difference of 28.6% NDF. This could lead to a difference in how divergent the microbial populations of animals consuming the respective forages are. The respective microbial populations of animals consuming grass silage or corn silage were adapted to numerically similar amounts of NDF, and so no differences in NDF digestibility were found (Boguhn et al., 2013).

Furthermore, the ADF content of the grass silage (27.3%) and corn silage (22.5%) with a difference of 4.8% ADF used by Boguhn et al. (2013) was greater than the difference of NDF of

the grass and corn silages and could have been a contributing factor to the difference in ADF digestibility found by the researchers.

The significant effect in the current study of donor animal diet on *in vitro* fermentation results of alfalfa hay could be a result of the plant's cell wall composition. Due to the higher level of intake driven by lower NDF content, the rate of passage of alfalfa hay through the digestive tract is greater than bermudagrass. With a high rate of passage, the microbial populations of horses consuming alfalfa hay must be accustomed to rapidly attaching to forage particles and digesting the available nutrients despite the more highly-lignified cell wall (Hainze et al, 2003). It is also important to note that the differences in CP and TNC content of the two forages may have impacted the microbial population makeup as well. The high CP content of the alfalfa hay (18.67%) compared to the bermudagrass hay (7.72%) may have influenced the hindgut microbiome and had an impact on the results of the current study. The TNC content of the alfalfa and bermudagrass hays were 8.32% and 12.51% respectively. The TNC procedure detects the NSC and starch fractions of forage. These fractions are fermentable by microbial organisms and thus alter the abundance of fermentable substrate present within each incubation vessel. These differences in CP and TNC content of the forages evaluated may have altered the microbial population makeup and therefore altered DM, NDF, and ADF digestibility estimations.

However, when bermudagrass hay samples were fermented, the inoculum source had no effect on DM, NDF, or ADF digestibility estimations at 48 of fermentation. By 72 h of fermentation, however, DM digestibility estimations were higher with alfalfa-sourced microbial inoculum. Because bermudagrass is often less digestible than alfalfa, there exists less of an opportunity for the microbial inocula to digest the bermudagrass hay samples to a different extent.

Earing et al. (2010) reported that after 72 h of *in vitro* fermentation in fecal microbial inoculum sourced from animals consuming the diet under evaluation are not different from *in vivo*. Based on those findings, incubating a feedstuff in the current study with a microbial inoculum obtained from an animal consuming a different feedstuff leads to inaccurate estimations of the potential extent of *in vivo* digestibility. In the case of alfalfa hay, bermudagrass-sourced fecal inoculum lead to significantly lower estimations of digestibility of DM, NDF, and ADF at both fermentation times. For bermudagrass hay, the effect was less consistent, but alfalfa-sourced fecal inoculum tended to yield lower estimations of NDF digestibility ($P = 0.804$) at 48-h incubation times. Additionally, alfalfa-sourced fecal inoculum produced greater DM digestibility estimations at 72-h incubation times, and a trend toward greater estimations NDF digestibility ($P = 0.0544$) at 72 h of fermentation. These results may lead to an overestimation of *in vivo* digestibility values.

CONCLUSIONS

The objective of the present study was to evaluate the effect of donor animal diet on *in vitro* fermentation digestibility estimates. Using fecal microbial inoculum from equids consuming two different diets, hay samples were fermented, with DM, NDF, and ADF digestibility estimations compared across inoculum types.

Digestibility estimations were significantly different for alfalfa hay samples, with digestibility estimations being consistently greater when fermentation was carried out with alfalfa-derived fecal inoculum at both 48- and 72-h incubations. Digestibility estimates were not different for bermudagrass hay samples when incubated with either alfalfa- or bermudagrass-derived fecal inoculum after 48 h of fermentation. However, DM digestibility of bermudagrass hay was greater when incubated with alfalfa-sourced fecal inoculum for 72 h.

Based upon these results, forage diets consisting of different types of forage may yield different *in vitro* digestibility estimations. The possibility for over- or underestimation exists and should be avoided by adapting inoculum donor animals to the feedstuffs being evaluated. Results from the current study imply that horses should be adapted to the forage diet that will be evaluated *in vitro* if possible. Despite adaptation to the forage diet being required for the most accurate digestibility estimations, *in vitro* fermentations hold advantages over *in vivo* estimations. *In vivo* estimations still require the adaptation of the animal to the diet being evaluated, and an additional period of several days in which a total fecal collection must be performed. Such *in vivo* methods can be costlier to perform, cause a greater impact on the animal's freedom, and require a greater input of both human and forage resources.

If samples of a novel diet are not available in large enough quantities for adaptation of the animal, care should be taken that all samples are fermented using the same inoculum source to

avoid unnecessary variation of fermentation results, and the donor animal diet should be of a similar category as the novel feedstuff(s) under investigation. The results of this study indicate adaptation of inoculum donor animals to the all-forage diet under evaluation increases the accuracy of *in vitro* digestibility estimations when equine fecal microbial inoculum is used.

Research conducted in the future should be performed with a larger number of horses, and should incorporate different species and varieties of forages. The current study utilized a cool-season legume and a warm-season grass to determine whether radically different forages would elicit a change in *in vitro* digestibility estimations performed using equine feces as an inoculum source. It is impossible to predict whether similar species of forages, such as a cool- and warm-season grass, would yield similar results.

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APPENDIX

Appendix 1. Guaranteed analysis of trace mineralized salt block

Mineral	Amount	
Calcium (Ca)	Minimum	1.80%
	Maximum	2.80%
Salt (NaCl)	Minimum	90.00%
	Maximum	95.00%
Sulphur (S)	Minimum	1.00%
Cobalt (Co)	Minimum	25 ppm
Copper (Cu)	Minimum	150 ppm
Iodine (I)	Minimum	90 ppm
Iron (Fe)	Minimum	1500 ppm
Manganese (Mn)	Minimum	3000 ppm
Selenium (Se)	Minimum	10 ppm
Zinc (Zn)	Minimum	2500 ppm

Appendix 2. Equations used in the calculation of % DM, NDF, and ADF and % loss

Parameter	Equation
% DM	$\left[\frac{dry\ sample\ wt}{initial\ sample\ wt}\right]$
% DM loss	$\left[\frac{initial\ dry\ wt - final\ dry\ wt}{initial\ dry\ wt}\right]$
% NDF	$\left[\frac{(dry\ sample\ and\ bag\ wt\ after\ NDF) - sample\ bag\ wt \times \frac{final\ C^{\dagger}\ bag\ wt}{initial\ C\ bag\ wt}}{dry\ sample\ initial\ wt}\right]$
% NDF loss	$\left[\frac{NDF\ of\ C - NDF\ of\ sample}{NDF\ of\ C}\right]$
% ADF	$\left[\frac{(dry\ sample\ and\ bag\ wt\ after\ ADF) - sample\ bag\ wt \times \frac{final\ \mathrm{C}\ bag\ wt}{initial\ C\ bag\ wt}}{dry\ sample\ initial\ wt}\right]$
% ADF loss	$\left[\frac{ADF\ of\ C - ADF\ of\ sample}{ADF\ of\ C}\right]$

[†]Control

Appendix 3. Calculating total nonstructural carbohydrate (TNC) on % DM basis given titer amount and standard curve for the batch of Shaffer-Somogyi reagent used

1.	[Weight of sample (g) × DM] × 1000 = DM (mg)
2.	Enzyme blank titer (mL) – Sample titer (mL) = Adjusted titer (mL)
3.	[(Slope of standard curve × 0.01) × Adjusted titer (mL)] + (Y-intercept of standard curve × 0.01) = Equation derived number
4.	Equation derived number × 25 = Dilution correction
5.	Dilution correction / DM (mg) = % TNC on DM basis

All % TNC figures should be calculated in duplicate and analyzed for coefficient of variation to lie below 5