2018

APPLICATIONS IN UTILIZATION OF FORAGE CHEMICAL COMPOSITION AND PREDICTING EQUINE DIGESTIBILITY

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Digital Object Identifier: https://doi.org/10.13023/etd.2018.357

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APPLICATIONS IN UTILIZATION OF FORAGE CHEMICAL COMPOSITION AND PREDICTING EQUINE DIGESTIBILITY

THESIS

A thesis submitted in partial fulfillment of the

requirements for the degree of Master of Science in the

College of Agriculture, Food and Environment at the University of Kentucky

By

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2018

ABSTRACT OF THESIS

APPLICATIONS IN UTILIZATION OF FORAGE CHEMICAL COMPOSITION AND PREDICTING EQUINE DIGESTIBILITY

Most forage quality models were developed for ruminant nutrition, and may not apply to the horse. This two-part study evaluated the relationship between forage chemical composition and dry matter digestibility (DMD) using an in vitro method with equine feces as the inoculums. The first experiment determined that compared to 48 h of incubation, 72 h of incubation resulted in higher DMD for some forages. As a result of experiment 1, incubations in experiment 2 were conducted using 48 and 72 h incubation periods at 38 degrees C. The second experiment evaluated the effect of chemical composition on DMD. Thirty-one hay samples were used that ranged from 33% to 71% for NDF, 21% to 44% for ADF and 6.7 to 25.6% for CP (all on DMB). There were inverse relationships between ADF and DMD (r = -0.826 at 48 h; -0.841 at 72 h) and NDF and DMD (r = -0.779 at 48 h; 0.812 at 72 h). There was a positive relationship between CP and DMD (r =0.572 at 48 h; 0.615 at 72 h). Forage chemical composition, particularly ADF and NDF, has potential to predict digestibility of forages by horses.

KEYWORDS: in vitro, forage, digestion, chemical composition, horse

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06/07/2018

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For my community of friends, family,
and furry friends
that supported me through this journey.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Laurie Lawrence, for her guidance, patience, and advice during these projects. Without her support, I would never have had the opportunity to be here. I would also like to thank my committee members Dr. Bob Coleman and Dr. Ray Smith for their time, support and contributions to my thesis. I would also like to thank Dr. Steve Isaacs, Kristen Wilson, and Dr. Merlin Lindemann for the support and encouragement during my time in graduate school. I greatly appreciate the Pasture Evaluate team of Dr. Ray Smith and Krista Lea for sparking my initial interest in forages, as well as Dr. Ben Goff for continuing my interest in forage research as an undergraduate.

Thank you to my family for encouraging and supporting me through this process. I would not have achieved what I have without you. Many thanks to my friends and 'faith family' that helped me to keep going when I needed it, and gave me a community to be loved and accepted. Thank you to Ms. Alice and Cathy Dibben of Sandhill Horse Farm for sparking my initial love for horses. Finally, thank you to the horses that so willingly gave samples so this study could happen.

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Chapter 1: Introduction

A study surveying the feeding practices of horse owners found that although almost all owners fed forages of some type, only 21% of forages fed were analyzed and only 5 horses out of the 337 horses in the survey had free access to forages (Hoffman et al., 2009). Hay is often the most popular forage for horses (Wylie et al., 2013), however most horse owners do not consider the chemical composition of the hay purchase, but rather evaluate forages from physical characteristics, and buy based from regional availability (USDA, 1998; Stapper, 2011). Furthermore, horse owners may end up paying more for a lower quality hay compared to ruminant producers (Grisley et al.,1985). These findings indicate a lack of understanding on the part of horse owners in terms of how to assess forage quality and its impact on horse feeding practice. Models to assess forage quality have been developed for ruminants however similar models are yet to be clearly determined for horses (Pearson et al., 2006). To better advise horse owners, more information is needed to understand how chemical composition of forages might be used to model forage quality for horses and provide a more useful evaluation tool for horse owners

Chapter 2. Literature Review

Equine Digestion

Horses procure food through prehensile lips that allow them to sort through small feed particles, and grasp forages. The two rows of incisors allow them to graze closely as they can rip and bite closer to the ground than cattle. The tongue moves forages within the mouth to allow for grinding between molars and premolars, ensuring feed particles are thoroughly ground (Frape, 1999). Chewing breaks apart the forage particles, exposes

parts of the plant and creates smaller pieces that can then be further digested as they move along the gastrointestinal tract.

Horses with well-formed teeth usually will break down hay particles to less than 1.6 mm in length, which are then further reduced to almost 1 mm in the horse's stomach (Frape, 1999). Breaking forage particles down takes time; horses spend more time chewing hay compared to any other feedstuff (Ellis and Hill, 2005). When horses chew, they release saliva which lubricates feed boluses to assist in swallowing, and provides some buffering of stomach acids (Nadeau et al., 2000).

The mouth also plays a role in selection of feedstuffs. Chemoreceptors on the tongue play a role in selection or avoidance of certain flavors. Horses have sensitive tastes, particularly regarding novel feeds. Studies evaluating taste in the horse have found they prefer sweeter flavors over sour, salty, acidic or bitter (Randall et al.,1978). As horses browse forages, they can be selective of what they choose to consume.

Once chewed, feed particles become a bolus, or small ball of feedstuffs that is swallowed and then passes through the esophagus. The esophagus is approximately 150 cm in length and plays no role in digesting nutrients other than acting as a conduit to the stomach. The horse's stomach is simple compared to the quadruple segments of the ruminant stomach, and makes up only ten percent of the total adult gastrointestinal tract (Frape, 1999).

The equine stomach has a non-glandular region and a glandular region. Feed exits the esophagus and enters the non-glandular region. The non-glandular region has a small role in digestion but because it lacks a mucous layer, it is highly susceptible to ulceration when it encounters secretions from the glandular region.

The glandular region is the lower portion of the stomach and is where digestive secretions occur. The stomach lining is filled with small pits containing parietal and chief cells which produce hydrochloric acid (HCl), pepsinogen, and mucous. The HCl creates a very acidic environment and activates pepsinogen, previously inactive as a zymogen, which then becomes pepsin, thereby initiating protein digestion. The cardiac glands within this region secrete bicarbonate and mucus which act as buffering agents and help to protect the gastric lining from ulceration (Singer, 1998). Feed particles are stored in the stomach of the horse for a very short period, 2 to 3 hours on average (Frape, 1999), so most digestion occurs later in the gastrointestinal tract.

The small intestine is approximately 21 to 25 m in length in the horse and is made up of three sections: the duodenum, jejunum and ileum (Frape, 1999). As the stomach slowly releases feed particles, it creates pockets of digesta moving through the intestines. As the chyme mixture moves through the intestine, digestion continues and absorption begins. The internal lining of the small intestine creates micro-pockets within finger-like projections known as villi. The villi help to slow the passage of digesta through the intestine, giving enzymes and other digestive properties time to facilitate the release of absorbable nutrients. The liver and pancreas, accessory organs in the digestive tract, release bile, mucous and digestive enzymes into the mix to start the next phase of digestion within the duodenum. Digesta leaving the stomach is extremely acidic due to HCl secretion within this region. Glands within the duodenum continue to secrete bicarbonate to buffer the pH throughout the digestive tract (Ellis and Hill, 2005). The villi also have deep pits, known as Crypts of Lieberkühn, that secrete digestive enzymes and

mucus to aid in the digestive process as digesta passes through the projection (Ellis and Hill, 2005).

Some of the protein in forages will be digested to di-peptides and then amino acids and absorbed in the small intestine. However, it appears that the amount of forage protein that is digested and absorbed varies among forages (Gibbs et al., 1988). Protein that is not digested in the small intestine will pass to the large intestine where it will become available to the resident microbial community there.

Starches are acted on by amylase, to break the polysaccharide into simpler sugar molecules (Ellis and Hill, 2005). The smaller sugar molecules can then be further digested through hydrolysis along the exterior of the villi projections, known as the brush border membrane, to create sugar monomers that can be absorbed through the intestinal wall (Gray, 1992). Other nutrients will also be absorbed from the small intestine, including lipids, various vitamins, and many minerals.

As chyme passes through the small intestine, there are no enzymes secreted by the horse that can act on the fibrous material. Forages contain a variety of fibers including cellulose, hemicellulose, pectin and fructans which will undergo minimal digestion in the stomach and small intestine. However, these compounds are susceptible to fermentation by the microbial community in the large intestines. Microbial fermentation of fiber in the hindgut produces volatile fatty acids (VFAs). These VFAs are then absorbed by the horse and used in energy production pathways. B vitamins, vitamin K and proteins are also synthesized through the digestion of forages by microbes in the large intestine (Pilliner, 1999).

Forage also plays an important role in helping to maintain the microbial community of the equine gut. The gastrointestinal microbiome is of importance as it is related to both gut function, as well as overall health in the horse (Costa and Weese, 2012). The microbiome is made up of a diverse microflora of bacteria, protozoa, archaea, and a limited number of fungi (Dougal et al., 2012). Although the microbiome is influenced by diet, several recent studies have aimed to evaluate the main components of the equine microbiome independent of diet, as well as how diet affects changes within microflora (Costa and Weese, 2012; O'Donnell et al., 2013; Proudman et al., 2014). The gut microbiota has been shown to be predominantly made up of *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria, Euryarchaeota, Fibrobacteres* and *Spirochaetes*; with smaller amounts of *Fibrobacter, Faecalibacterium, Ruminococcus, Eubacterium, Oscillospria, BlautiaAnaerotruncus, Coprococcus, Treponema* and *Lactobacillus* spp. (O'Donnell et al., 2013).

The microbiome in the horse is largely dependent on the environmental conditions such as substrate availability and pH. Recent studies have found that when the pH of the large intestine falls below 6.0, the number of acidophiles increases while the number of fiber-fermenting microbes decreases (Milinovich et al., 2008; Biddle et al., 2013). Biddle et al. (2013) suggest that diets that emphasize starch lead to a change in the VFA profile and lactic acid production, thereby reducing pH which if sustained over time, can irritate and potentially damage the intestinal lining, potentially increasing the risk of colic, and/or laminitis. Forage based diets provide a balance of non-structural carbohydrates and structural carbohydrates (aka fiber), helping to maintain a normal gut microbiome and pH.

Forages for Horses

Horses consume forages in many forms. Grazing animals will consume fresh forage however when fresh forage is not available, some type of conserved forage will be needed. In other countries, ensiled forages may be used for horses, but in the U.S. hay is the most common conserved forage used for horses. Horse owners may purchase hay based on sensory criteria such as color and smell, or by type. The most common hay types are legumes such as alfalfa (*Medicago sativa*), cool season grasses such as timothy (*Phelum pretense*), orchardgrass (*Dactylis glomerata*), smooth bromegrass (*Bromus inermis*) and warm season grasses such as Bermudagrass (*Cynodon dactylon*). Mixed hays containing a combination of different forage types are also common.

Hay type is one of the factors that has a significant effect on forage chemical composition. Forage chemical components are divided into cellular contents and cell wall fraction (Van Soest, 1967). Cellular contents make up the fraction of forages easily digested by the horse, such as sugars, soluble carbohydrates, proteins, etc. Cell wall components typically have lower digestibility and are made up of hemicellulose, cellulose, lignin and heat-damaged protein (Van Soest, 1967). The cell wall components are indigestible by the horse alone and must be digested through microbial enzymes, as described earlier.

The effect of plant type on chemical composition has been studied for more than 50 years. For example, in 1967 Fonnesbeck et al. discussed the dry matter components of forages that were determined through various systems of analysis available at that time. Grass forages were found to contain larger amounts of crude fiber compared to alfalfa. Furthermore, Fonnesbeck et al. (1967) stated how the composition of fiber differs

between grass and alfalfa forages which was illustrated through the evaluation of the cell wall constituents. Grass forages had greater amounts of holocellulose (hemicelluloses and cellulose), accounting for the larger crude fiber values, whereas the alfalfa forages had larger amounts of lignin (Fonnesbeck et al., 1967). Cellulose was found to be similar between grass and alfalfa forages.

Today, crude fiber is not commonly used in forage analysis. Instead forage analyses generally list two fiber fractions, the neutral detergent fiber fraction (NDF) which contains hemicellulose, cellulose and lignin and the acid detergent fiber fraction (ADF) which contains cellulose and lignin. The amount of NDF and ADF in hay is affected by forage type. Legumes tend to be lowest in NDF and warm season grasses highest in NDF. However, legumes generally contain lower concentrations of hemicelluloses than grasses. Legumes also tend to be higher in other components, including protein and calcium compared to grasses.

Another factor affecting forage chemical composition is stage of maturity of the plant at harvest. Plant maturity plays an important role in carbohydrate ratios within the plant. Stage of plant maturity when the forage crop is harvested affects the degree of fibrous material and quality (Van Soest, 1967). As plants mature, the fibrous fraction increases with increased lignification due to the increase in structural components (i.e. stem and branching). Table 2.1 shows the effect of stage of maturity on the chemical composition of alfalfa hay and grass hay.

The relationship of forage digestibility to forage chemical composition

As discussed above, both plant type and stage of maturity at harvest can influence concentrations of crude protein, NDF and ADF. These differences in composition,

especially the fiber fraction influence digestion in the horse. Vander Noot and Gilbreath (1970) first evaluated these differences by comparing digestibility values for several different forages by geldings and steers. They used 4 different forages - alfalfa, timothy, smooth bromegrass, and orchardgrass - which varied in chemical composition. They found differences in forage utilization between cattle and equids, but more importantly, they observed differences in how well the horses digested alfalfa, compared to orchardgrass, timothy or bromegrass hays.

Fonnesbeck et al. (1967) also evaluated differences in digestibility of forages by horses in a two-part experiment. In the first part of the study he used smooth bromegrass, timothy, "Alta" tall fescue (*Fesctuca arundinacea),* reed canarygrass (*Phalaris arundinacea*), "Atlantic" alfalfa, and "Pennscot" red clover (*Trifolium pretense*). The second experiment used Alta tall fescue (*Fesctuca arundinacea*), reed canarygrass, Atlantic alfalfa, "Lincoln" smooth bromegrass, orchardgrass, and "Midland" bermudagrass. Forages in this study ranged in chemical composition. The % CP ranged from 8.3 to 14.2 in experiment 1 and from 8.3 to 16.0 in experiment 2. The % crude fiber ranged from 29.5 to 44.1 in experiment 1, and from 30.6 to 37.9 experiment 2 (Fonnesbeck et al. 1967). In both parts of the study, alfalfa was more digestible than the grass forages (Fonnesbeck et al., 1967). Even the alfalfa described in the study as being a more mature and stemmy hay, was more digestible than grass forages. Fonnesbeck et al. (1967) also reported the chemical composition of the forages. In addition to differences in cell wall components between grasses and legumes, clear differences in soluble carbohydrates were also observed between forage types with legumes having a larger portion of soluble carbohydrates compared to grasses (Fonnesbeck et al., 1967). The

difference between soluble and insoluble (fibrous) plant carbohydrates is an important component in forage quality for horses. Horses, being non-ruminants, can utilize soluble carbohydrates in the foregut, with insoluble or fibrous carbohydrates processed through microbial fermentation the hindgut. Alfalfa, despite having a higher level of lignin compared to grass forages, overall still had a larger soluble carbohydrate composition and higher digestibility. Fonnesbeck et al. (1967) also reported that in the first part of the study, alfalfa had higher digestibility of crude protein while the other legume, red clover, had more total digestible nutrients (TDN) and digestible dry matter compared to the grasses. But timothy and bromegrass had higher digestibility of crude fiber compared to alfalfa, red clover, and canarygrass (Fonnesbeck et al., 1967). These results were repeated in the second portion of the study with alfalfa hay having consistently higher digestibility than grass hays (Fonnesbeck et al., 1967).

Grass forages were similar in fiber digestibility, dry matter digestibility, and nitrogen free extract (NFE) digestibility. However, there were differences among grasses in regard to protein digestibility. The grass hays with higher crude protein concentration also had the highest protein digestibility (Fonnesbeck et al., 1967). In contrast, when legumes were compared in experiment one, alfalfa, despite have a lower crude protein content compared to red clover, was more digestible (Fonnesbeck et al., 1967).

Overall, horses can digest more DM from legume hays, compared to grass hays which are generally higher in fiber and lower in crude protein (Fonnesbeck et al., 1967; Darlington and Hershberger, 1968; Vander Noot and Gilbreath, 1970; Cymbaluk and Christensen, 1986). Forage fiber content is also influenced by stage of maturity. Young, immature plants are higher in the leafy material as the stem and branching portions of the

plant are less developed and therefore the plant is less fibrous making it more digestible to the horse. As the plant matures, fiber and lignin concentrations increase, reducing forage digestibility (Yari et al., 2017).

Measuring Forage Digestibility

Forage digestibility by animals can be evaluated by several methods. The initial methods used in vivo animal digestibility trials. Animals would be adapted to consuming the forage of interest for several days or weeks and then forage intake and total fecal output would be measured for several days. The feed and feces would then be analyzed and digestibility determined by difference. These types of experiments involve many animals, are time consuming and expensive to conduct. In situ methods have also been used, often with cannulated animals. Forage samples are weighed into porous bags which are then introduced into the animal's digestive tract. After removal the bags are dried and reweighed to determine disappearance of the forage and thus digestibility. Although in situ methods may return data more rapidly than total fecal collection studies, cannulated animals may require long term care and can be very expensive. Due to the expense and labor of these types of nutrition studies, in vitro methods for estimating digestibility have become more popular.

In 1963, Tilley and Terry published a full method using a two-stage in vitro method to evaluate the digestibility of forages. To evaluate forages through this method, samples are obtained and either frozen with liquid nitrogen or dried, ground through a 1 mm screen, and digested first in a fermentation step involving rumen fluid and then in an enzymatic step using pepsin (Tilley And Terry, 1963). This method used individual tubes for each sample and more recently batch systems have been developed for measuring in

vitro digestibility. One of these batch methods utilizes a commercially available system, the Daisy II (ANKOM Technologies). The Daisy II was developed to evaluate many samples at the same time using rumen fluid as the inoculant. The system includes 4 large incubation vessels that can hold approximately 25 porous bags with feed samples. The vessels are incubated in the temperature controlled Daisy II cabinet.

These in vitro methods were developed using rumen fluid and therefore are specific to predicting forage digestibility by ruminants. However various efforts have been made to develop an in vitro method for predicting forage digestibility by the horse. An obstacle to developing in vitro models of equine digestion is the difficulty in obtaining and maintaining donor animals that could provide cecal or large colon fluid as inoculum. Consequently, Akhter et al. (1999) and Lowman et al. (1999) determined that feces can be effectively used as a source of microbial inoculum for in vitro digestibility determinations. In 2007, Lattimer et al. determined that equine feces could effectively be used in the Daisy II Incubator. Most recently, Earing et al. (2010) extensively evaluated the differences between in vitro digestibility in the Daisy II and in vivo forage digestibility, through the assessment of two forage types, timothy and alfalfa, in diets supplemented with or without oats. The study found that in vitro measures of digestibility using the Daisy II incubator with fecal inoculums were highly correlated to in vivo results.

Assessing Forage Quality- a producer perspective

Forage scientists have attempted to simplify the evaluation of forage quality using chemical composition by developing forage quality indices. One index is relative feed value (RFV) which was designed for marketing of hay crops (Moore and Undersander,

2002). Using the NDF and ADF concentrations of the forage RFV estimates the digestible dry matter (DDM) of as well as the dry matter intake (DMI) of the hay by cattle to give a general assessment of feeding value. The calculation of the formula is shown below:

$$DMI, \%\ of\ BW = \frac{120}{(NDF, \%\ of\ DM)}$$

$$DDM, \%\ of\ DM = 88.9 - 0.779 \times (ADF, \%\ of\ DM)$$

$$RFV = \frac{DMI \times DDM}{1.29}$$

The formula is based off comparing a forage sample to full bloom alfalfa, which is given an RFV of 100, resulting in the 1.29 denominator value. Due to this comparison, all forages are categorized against full bloom alfalfa, thereby ranking forage quality. Theoretically, the index relates the price hay should be sold at by sorting hay in terms of the value to animal feeding and production. However, in this index all calculations are based on data derived from studies with ruminants and the application of RFV to equine feeding is unknown.

Relative feed quality, RFQ, is another means of ranking forage quality. RFQ is different from RFV as it also takes fiber digestibility, or fiber profile, into account. Forages tend to group similarly in RFV and RFQ when of high quality (i.e. similar low levels of fiber), but for lower quality forages, meaning those higher in indigestible fiber content, RFQ values are typically lower and can differ greatly depending on forage type and maturity. The formula for RFQ, calculated through the use of TDN, is shown below (Moore and Undersander, 2002).

$$RFQ = \frac{(DMI, \%\ of\ BW) \times (TDN, \%\ of\ DM)}{1.23}$$

$$TDN = tdCP + (tdFA \times 2.25) + tdNDF + tdNFC - 7$$

$$tdCP = total\ digestible\ CP,$$

$$tdFA = total\ digestible\ fatty\ acids,$$

$$tdNDF = total\ digestible\ NDF,$$

$$tdNFC = total\ digestible\ nonfibrous\ carbohydrates$$

Although RFQ better models the fiber digestibility, it is still largely developed from ruminant research. Anatomical differences between horses and ruminants have lead many equine researchers to question the validity of applying RFV or RFQ equations to predicting the value of forages for horses.

Several researchers have evaluated the digestive differences between horses and ruminants. Cymbaluk et al. (1990) evaluated differences in digestion among six specific forages fed to both cattle and horses. Intake was found to be different by type of forage between cattle and horses, and the ability to predict intake from composition was also different between cattle and horses. These differences in intake, and prediction of intake, could affect the usefulness of both the RFV and RFQ formulas when predicting forage value for horses. Overall, cattle digested more dry matter (DM), gross energy (GE), fiber (ADF/NDF), and P from hay compared to horses when fed a variety of hay types. However, when fed alfalfa alone, horses and cattle digested energy and fiber content similarly (Cymbaluk et al., 1990). Additionally, when fed higher quality forage types, such as alfalfa, horses digested more CP from certain hay types, when compared to cattle

(Cymbaluk et al., 1990). Thus, it appears that horses and cattle digest higher quality (lower NDF, higher CP) forages equally well. However, Cymbaluk et al. (1990) found cattle have a higher capacity to digest lower quality forages (higher NDF, lower CP) than horses.

These differences in digestion may be due in part to the anatomical differences between ruminants and horses (non-ruminants), leading to differences in digestion of forages. One of the main differences between the two is digestibility of fiber (NDF/ADF). Ruminants have a four-compartment stomach where microbial digestion occurs prior to any digestion by endogenous enzymes, leading to a slightly higher proportion of cellulolytic activity in cattle (Cymbaluk et al., 1990). Koller et al. (1978) used both in situ and in vitro techniques and found that the digestibility of high quality alfalfa was similar between cattle and horses, but that grass forages were less completely digested by horses compared to cattle. Those authors found differences even when incubation times were similar across animal species.

These differences may be due to differences in in vivo retention times; the GI anatomy of ruminants results in longer retention time of particles in the GI tract. Pearson et al. (2006) found that the mean retention time (MRT) within the gastrointestinal tract was generally shorter in equids compared to ruminants, but varied slightly depending on type of forage. Mendoza et al. (2016) found that dairy cattle typically have a MRT between 31 and 39 hours, which can be affected by level of forages present in the diet. Earing et al. (2013) reported that the total tract mean retention time for the particle phase of digesta in horses was 24.9 hours. Overall, ruminants are able to digest lower quality forages more effectively than horses, either due to differences in the microbial community

of the GI tract or as a result of longer particle retention in the GI tract. Consequently, forage indices based on ruminant digestibility studies may accurately estimate the value of high quality forages for horses, but may overestimate the value of lower quality forages for horses.

Very few in vivo or in vitro studies have systematically evaluated the relationship between forage chemical composition and forage digestibility in horses. Hansen and Lawrence (2017) reviewed published research studies regarding forage digestion in the horse, focusing on digestibility differences due to chemical composition differences. Through simple linear regression of the data, they found that all forage composition factors, including NDF, ADF and CP, were significant factors in predicting forage digestibility in the horse. Several regression equations were evaluated and the final equation that was thought to best predict digestibility was best explained through using two factors: NDF and CP. The two-variable equation developed by Hansen and Lawrence (2017) is shown below:

$$DMD = 65.81 + 0.7207 \times CP - 0.3514 \times NDF$$

This research helped to set a significant foundation for better predicting forage digestibility in the horse. Although the study was able to use a wide range of forage types and data, in vivo results were collected from various studies, where differences among study methods could lead to variability in results. The authors also suggested that the use of forages with a wider range of chemical composition and more similar methods to measure dry matter digestibility could lead to a more robust prediction model. Because in vivo digestibility studies are labor and animal intensive, in vitro digestibility assays may

provide additional information on the relationship between forage chemical composition and dry matter digestibility of forages by horses.

Towards an Equine Forage Quality Index

Traditionally, producers have used physical or sensory variables to evaluate forage quality. These variables typically encompass forage color, presence of leaf material, absence of foreign material/debris, smell of forage, and the absence of mold (Gibbs, 2005). Horse owners may be able to judge forages in terms of physical quality; however, they may find the relationship between forage quality and chemical composition variables such as NDF or ADF, more difficult to interpret effectively.

As described previously, there is good evidence that forage chemical composition, particularly crude protein and fiber content, will affect digestibility and thus nutritive value. Consequently, the main focus of the research conducted in this thesis will be related to that relationship. However, regardless of how digestible a forage may be, it does not have good feeding value unless the animal consumes it.

Thus, it is important to consider that another factor affecting feeding value is voluntary intake. Equids can be selective with what they eat, sorting through hay and forages to consume 'more desirable' components. The preferences of animals for various forages or forage components is an area of particular interest as it may affect voluntary dry matter intake (VDMI) (Crampton, 1957; Minson, 1982; Van Soest, 1983; LaCasha, 1999). It has been suggested that the voluntary intake of forages by animals can be influenced by forage chemical composition, palatability, physical characteristics, as well as animal factors such as digestibility and rate of passage (Minson, 1982; Van Soest, 1983).

Several studies have been done in recent years to evaluate if there is any connection between forage chemical composition and equine preference or voluntary intake. Rodiek and Jones (2012) evaluated four forages including alfalfa, teff (*Eragrostis tef*), oat (*Avena sativa*), and wheat hay (*Triticum aestivum*), finding that intake was greatest for alfalfa followed by teff, wheat and then oat hay. The horses consumed enough of the alfalfa hay to meet most nutrient needs. They consumed enough teff to meet all nutrient requirements except digestible energy, but did not consume enough of the wheat and oat hay to meet the nutrient requirements evaluated (Rodiek and Jones, 2012). In rank order, the horses consumed alfalfa, teff, wheat and oat hay, while the CP concentrations for those hays were 23%, 19.7%, 8.7% and 8.7%, respectively. Alfalfa, which had the highest intake, also had the lowest concentrations of NDF and ADF.

In a similar study, Crozier et al. (1997) found that horses consumed more alfalfa than tall fescue and Caucasian bluestem (*Bothriochloa bladhii*), with no difference in intake between the grasses. When forage chemical composition is compared, that alfalfa hay was higher in crude protein and lower in NDF and ADF than the grasses. The alfalfa also contained more lignin, less hemicellulose and higher calcium, phosphorus, and iron (Crozier et al., 1997). When digestibility of these same forages was compared, not surprisingly, in vivo alfalfa DM digestibility and IVDMD were significantly higher than the grass hays (Crozier et al., 1997).

The geldings fed alfalfa in this study had higher absorption of calcium, potassium, sulfur, and a trend towards higher absorption of phosphorus compared to both grass hays, whereas fescue had higher absorption of magnesium, potassium and sulfur compared to the bluestem (Crozier et al., 1997). Blood variables were also different between horses

fed the different hays indicating potential differences in digestibility (Crozier et al., 1997). The geldings fed alfalfa hay had higher levels of blood urea nitrogen, vitamin A, phosphorus, and sulfur compared to horses fed both grasses, and those fed tall fescue had higher levels of blood urea nitrogen, selenium and zinc compared to bluestem (Crozier et al., 1997).

LaCasha et al (1999) aimed to further evaluate the connection between the forage chemical composition and intake by evaluating three different hays fed to yearling horses. Yearling horses have larger nutrient requirements due to their fast growth and development. The researchers fed "Matua" prairie bromegrass (*Bromus kalmii*), "Coastal" bermudagrass, and alfalfa over the study period and evaluated voluntary intake when horses were fed a single forage and forage preference when horses were given access to all three forages at one time (LaCasha et al, 1999). Voluntary intake was greatest for alfalfa at 10.9 kg/d, followed by the prairie bromegrass hay at 10.0 kg/d, and lastly the bermudagrass at 7.4 kg/d. When all three forages were offered at the same time, horses consumed more alfalfa, indicating a preference for alfalfa over the two grasses. Other studies support the equine preference and higher intake of alfalfa over grasses (Crozier et al., 1997).

There were also differences in preference and intake of the grasses. Prairie bromegrass was consumed significantly more than the bermudagrass hay despite the mean digestibility for the grasses having no significant difference (LaCasha et al., 1999). In addition, prairie bromegrass was also selected more compared to the bermudagrass, indicating a preference for the prairie bromegrass hay over bermudagrass. LaCasha et al. (1999) conjectured that the differences in preference and intake were due to differences in

forage chemical composition between the two grass hays. Alfalfa was highest in CP (20%) and lowest for NDF (36.5%) and ADF(30.3%). Prairie bromegrass was intermediate in CP (13.5%), NDF (62.4%) and ADF (36.1%). Bermudagrass had lowest CP (11.3%) and the highest NDF and ADF, 78.3% and 40.0% respectively.

Many nutritionists have suggested that there is a relationship between forage chemical components, digestibility, and feeding value of forages, however this relationship may be complex (Crampton, 1957; Aiken et al.,1989; Cunha, 1991). When forages are deficient in certain nutritional factors, animals might increase dry matter intake, thereby normalizing nutrient intake. However, Cunha (1991) reported that forages with lower protein levels often have decreased intake by horses which may explain why forages such as bermudagrass, and other grasses lower in crude protein have a low intake level. Minson (1982) found that forages below 6 to 8% crude protein can result in reduced intake by ruminants. In addition, very high fiber concentrations may limit intake as in most studies have found that higher VDMI is associated with the forages with lower fiber concentrations (Crozier, et al., 1997; LaCasha et al., 1999; Staniar et al., 2010).

The findings of these experiments indicate a connection between forage chemical composition and forage intake or forage preferences in horses. When combined with the relationship between chemical composition and forage digestibility, it may be possible to develop a single, easy to understand, index that horse owners can utilize in the selection of forages for various classes of horses.

In vivo studies will be needed to determine the relationship between chemical composition and intake; however, in vitro methods may be able to expand our understanding of the relationship between chemical composition and forage digestibility

by horses. The objectives of this thesis were to further develop the equine in vitro method of determining dry matter digestibility using the Daisy II incubator and then to assess the relationship between NDF, ADF and CP and in vitro dry matter digestibility.

Table 2.1 Effect of stage of maturity on composition of selected forages (DMB) *

Forage	Stage of maturity	CP (%)	ADF (%)	NDF (%)
Alfalfa	Bud	22-26	28-32	38-47
	Mid bloom	14-18	36-40	46-55
	Full bloom	9-13	41-43	56-60
Orchardgrass	Vegetative- Boot	12-16	30-36	50-56
	Boot- Head	8-12	36-42	56-62
Bermudagrass	4-week growth	10-12	33-38	63-68
	8-week growth	6-8	40-45	70-75

Source: Ball et al., 2007.

Chapter Three: The effect of incubation time and temperature on in vitro forage digestibility

Introduction

Using in vitro techniques to model digestion of animals has allowed advancements in animal nutrition. In vitro procedures can be done in a lab setting utilizing less time, and can be more economical. Tilly and Terry (1963) pioneered ruminant in vitro digestibility trials using rumen fluid as an inoculum source. This method was further developed for horses using cecal fluid (Trevor-Jones et al., 1991), and later equine feces (Lowman et al., 1999). To facilitate analysis of a larger number of samples a commercially available batch incubation system was developed (DAISY II, Ankom Inc) and validated against the Tilly and Terry method. Research completed by Earing et al. (2010), found that the DAISY II incubation system had potential as a means of comparing in vitro digestibility of different diets in the horse using equine feces as an inoculum.

Although 24 and 48 h incubation periods have often been used in ruminant studies, Earing et al. (2010) investigated the impact of varying lengths of in vitro incubation periods and found that incubations of 40 h or less produce digestibility values lower than in vivo digestibility of the same feedstuffs (Earing et al., 2010), suggesting that longer incubation periods may be necessary to obtain complete digestion of some forages in vitro. In that study, an incubation temperature of 39^{o}C was used, based on the manufacturer's recommendations for rumen fluid. However, the core temperature of the mature horse is less than 39^{o}C, usually between 37^{o} and 38^{o}C, so incubation temperature could be another variable. The goal of this study was to examine the effect of incubation

time and incubation temperature on the in vitro dry matter digestibility (DMD) of four forages. The hypotheses were that DMD would be higher after 72 h of incubation compared to 48 h and that 38°C would also yield higher DMD values than 37°C.

Materials and Methods

Preliminary Study: Effect of number of Ankom Bags per vessel

A preliminary study was conducted prior to the main experiment to evaluate the effect of the number of bags per jar on DMD determinations. Four forages (Table 3.1) were utilized in this experiment: timothy hay, tall fescue hay and two alfalfa hays (alfalfa 1 and alfalfa 2). The samples were ground (1 mm) and then weighed into pre-dried Ankom F57 filter bags (0.4-0.5g). Once the filter bags were filled they were sealed, dried, and reweighed to determine the weight of each sample on a dry matter basis.

Feces collected from a pool of healthy, mature horses were used for inoculum. The horses were managed similarly at the University of Kentucky Maine Chance and housed together. Fecal samples were collected from whichever horse defecated first, gathered in large plastic bags to avoid contamination, and then placed in an insulated foam cooler to maintain microbe viability during transportation to the lab. Approximately 800 g of feces was needed so if one horse was not able to provide a large enough sample, a second sample was obtained from whichever horse in the pool provided feces next. The two samples would then be mixed together thoroughly once back in the lab.

Once at the lab, the feces were mixed and then weighed out at approximately 200 g into labeled mason jars (labeled respective to Daisy vessel) and stored in a water bath at 37°C while waiting to be mixed. Then, 400 ml of per-warmed incubation buffer was removed from the respective Daisy II vessel, added to the mason jar and mixed with a

blender to achieve a uniform, liquid consistency. The mason jar contents were then poured back into the Daisy vessel containing the filter bags and mixed buffer solution, and mixed. The pH of the vessel was taken prior to the start of incubation and adjusted, using the addition of Buffer A and Buffer B (Appendix 1) to achieve a final solution pH of approximately 7.

Two treatments of bag numbers were used: either 15 or 23 bags per vessel. The 15-bag set had triplicate bags of the 4 different hay samples and 3 blanks. The 23-bag set had the same 4 hay samples, but there were 5 bags per sample and a set of 3 blanks. Table 3.2 shows the mean and standard deviation for DMD of each hay when incubated in a 15-bag vessel or a 23-bag vessel. Although the mean DMD for each hay was similar in each vessel treatment, the standard deviation of the mean was smaller for 3 of the 4 forages in the 15-bag set compared to the 23-bag set. Ankom indicates that each vessel can accommodate up to 25 bags. However, that recommendation is based on rumen fluid as the inoculum, not feces. When feces are used, the material within each vessel is more viscous and mixing of bags may be less efficient, which could cause more variation among replicates. Subsequent experiments limited bag number to 15 per Daisy II vessel.

Main Experiment: Effect of incubation time and temperature on in vitro dry matter digestibility

The study used two temperatures (37^{o} or 38^{o}C) and two incubation periods (48 or 72 h) in a 2 x2 factorial design. The Daisy II incubator includes four, 2L incubation vessels in a temperature controlled cabinet; thus, each vessel had a different treatment combination. The experiment was repeated four times with treatments in different jar positions.

Four forages were utilized in this experiment: timothy hay, tall fescue hay and two alfalfa hays. Forages were selected to provide a range in chemical composition (Table 3.1). The samples were ground (1 mm) and then weighed into pre-dried Ankom F57 filter bags (0.4-0.5g). Each forage was weighed into 12 bags. Once the filter bags were filled they were sealed, dried, and reweighed to determine the weight of each sample on a dry matter basis.

Feces collected from a pool of healthy, mature horses were used for inoculum. The horses were managed similarly at the University of Kentucky Maine Chance and housed together. Fecal samples were collected from whichever horse defecated first, gathered in large plastic bags to avoid contamination, and then placed in an insulated foam cooler to maintain microbe viability during transportation to the lab.

Approximately 800 g of feces was needed so if one horse was not able to provide a large enough sample, a second sample was obtained from whichever horse in the pool defecated next. The two samples would then be mixed together thoroughly once back in the lab.

Once at the lab, the feces were mixed and then weighed out at approximately 200 g into labeled mason jars (labeled respective to Daisy vessel) and stored in a water bath at 37^{o} C while waiting to be mixed. Then, 400 ml of per-warmed incubation buffer was removed from the respective Daisy II vessel, added to the mason jar and mixed with a blender to achieve a uniform, liquid consistency. The mason jar contents were then poured back into the Daisy vessel containing the filter bags and mixed buffer solution, and mixed. The pH of the vessel was taken prior to the start of incubation and adjusted, using the addition of Buffer A and Buffer B (Appendix 1) to achieve a final solution pH

of approximately 7. This process was repeated for each Daisy vessel with 4 vessels per incubator. Each vessel was pumped with CO_2 for 30 seconds prior to being returned to the incubator for the assigned incubation time. Two incubators were used; one set at 37^{o}C and one set at 38^{o}C.

After the incubation period was over, post incubation pH was taken for each vessel, filter bags were removed and rinsed thoroughly in cold water until it appeared all fermentation residue was removed. Filter bags were then placed in a 100^{o}C convection oven to dry. After 24 h, the samples and bags were reweighed to determine DMD. The DMD results were compared within forage to evaluate the effect of time and temperature, and the interaction of time and temperature using the SAS PROC GLM procedure (SAS 9.4). Main effects and interactions were considered significant at $P<0.05$ and trends recognized at $P < 0.15$.

Results

The effects of incubation time and temperature on change in pH within the incubation vessels is shown in Table 3.3. Vessel pH was not different at the onset of the incubations between treatments. However, at the end of the incubations, vessel pH was lower, and the change in pH was greater in the 72 h incubations. There was no effect of temperature or an interaction between time and temperature on post-incubation vessel pH. The effect of incubation time and temperature on the DMD of each forage are shown in figures 3.1 – 3.4. There were no interactions between time and temperature for any forage ($P > 0.2$). However, there was a marked effect of incubation time on DMD of the tall fescue hay (figure 3.1; $P < 0.005$). Mean DMD for tall fescue at 48 h was 45.36% and increased to 54.1% at 72 h. Similarly, the DMD of the timothy hay (Figure 3.1) increased

from 48 to 72 h (54.3 to 56.7%, respectively), although the magnitude of the increase was not as great as for the tall fescue hay. Incubation temperature did not affect DMD for tall fescue but there was a trend for DMD to be higher for the timothy hay incubated at 38^{o}C.

There was a trend for both temperature and time to affect DMD for alfalfa-1 ($P < 0.1$). However, the mean DMD for samples incubated for 72 h had numerically lower values than the samples incubated for 48 h (68.7% vs 69.7%; Figure 3.3). For alfalfa-2, the effect of time was not significant ($P > 0.51$) but there was an effect of temperature ($P< 0.05$), with the 38^{o} incubation temperature producing a higher mean DMD (65.05%) than the 37^{o} incubation (63.9%).

Discussion

For this study, four different forages were used. Forages were selected to represent common hays used for horses, and to represent a range of qualities. Different forage types were selected to present a range of NDF, ADF, and crude protein concentrations. The two grass hay samples provided similar ADF and CP but differed in NDF with timothy having 59% NDF and the tall fescue having 67% NDF. The two legume hays were lower in NDF and ADF, but higher in CP content compared to the two grass forages. These two alfalfa hays were also selected to provide a range for legume hays with the first alfalfa having 36% NDF, 27% ADF and 19.3% CP compared to the second alfalfa sample which contained 44% NDF, 34% ADF and 17.4% CP. Earing et al. (2010) had previously suggested that lower quality forages might need a longer incubation period to achieve maximum DMD values. The goal in using forages differing in forage chemical composition was to evaluate if a difference in forage quality would produce results indicating a need for longer incubation periods.

The results indicate that forages higher in fiber, such as the tall fescue used in this study, may require longer incubation time to achieve the maximum DMD using DAISY II. These results are similar to the findings of Earing et al. (2010) where in vivo DMD values were best modeled with in vitro methods when incubation periods were greater than 40 hours. The two grass forages used in this study had higher DMD at 72 h of incubation compared to 48 h. Conversely, DMD was maximized at 48 h for legume hay as there was no significant difference between 48 h and 72 h of incubation on DMD of either alfalfa sample.

In this study, the incubation periods were much longer than would be expected in vivo. For example, mean retention time for feed particles in the equine gastrointestinal tract is about 25 h (Earing et al., 2013) and only a portion of that time would be associated with the large intestine. However, Earing, et al. (2010) had shown very low DMD values from in vitro data compared to in vivo values, when a 24 h incubation was used. These low in vitro DMD values obtained from 24 h incubations may be due to an initial lag period after starting incubation when microbes in the feces are starting to repopulate. However, it appears that by 48 h DMD is maximized for some forages, possibly those with lower NDF content. In the present study the two alfalfa hays had NDF concentrations below 45% while the two grass hays had NDF concentrations above 55%. Grasses, being higher in hemicellulose and cellulose, may need the additional time to properly be fermented with the inoculum and allow for breakdown of the fibers.

There was no significant effect of temperature from 37^{o}C to 38^{o}C for 3 of the 4 forages, but temperature did significantly affect one of the alfalfa hays in the sample set, with higher DMD in the 38^{o}C incubation. It may be important that two of the other

forages also showed a similar trend. As the effect of temperature was not significant for all hay samples, further research in this area is needed to definitively say if an increase in temperature would help to maximize DMD values. However, 38°C more closely approximates horse core temperature than the manufacturer's recommended temperature 39°C, so it may be a more relevant incubation temperature when using the DAISY II.

Differences in forage chemical composition may have influenced the DMD within each forage. The largest component of this is the fiber fraction. The larger the fiber fraction, the smaller the 'cell soluble' or easily digestible fraction of the hay. Alfalfa is known to have an overall lower fiber fraction compared to grasses which was consistent with the hays of this study as both alfalfa samples had a lower NDF content. Alfalfa's fiber fraction is made up of more lignin and lower amounts of hemicellulose and cellulose whereas grass forages are lower in lignin but higher in hemicellulose and cellulose fraction (Fonnesbeck et al., 1967). This is seen in the more mature, stemmy alfalfa sample, Alfalfa 2, with an ADF of 34%; very close to both the Timothy and Tall Fescue, despite having more than double the crude protein content. Interestingly, this alfalfa sample (alfalfa 2) was the only hay of the four that had a significant effect of temperature ($P<0.05$).

Further research is needed in this area to better predict digestibility based from forage chemical composition but the results of this study indicate that different forage types, grass forages, may need incubation greater than 48 h to allow for samples to fully digest.

Conclusion

With forages that vary in quality, greater incubation periods are needed with forage digested through in vitro methods. Further research is needed to determine if an increase in temperature influences digestibility of all forages types or only certain quality levels. Incubation periods over 48 h should be evaluated with the DAISY II incubation system to determine if they better model forage digestibility in the horse.

Table 3.1. Chemical composition of the forages used in the preliminary and main experiment in Chapter 3

Forage	NDF (%)	ADF (%)	CP (%)
Timothy	59.0	36.0	7.9
Tall Fescue	67.0	36.0	8.4
Alfalfa-1	36.0	27.0	19.3
Alfalfa-2	44.0	34.0	17.4

Table 3.2. Effect of number of bags in vessel on dry matter digestibility of four forages

Forage	Timothy	Fescue	Alfalfa 1	Alfalfa 2
15 Bags DMD (mean)	56.79	48.84	73.09	68.3
23 Bags DMD (mean)	56.76	50.29	73.97	68.63
15 Bags SD	0.4	4.01	0.42	0.3
23 Bags SD	1.15	2.56	0.74	1.15

*each DMD value is the mean of bag set (3 for 15, 5 for 23, with 3 blanks for each)

Table 3.3. Effect of incubation time and temperature on pH in incubation jars (mean +/- SE)

	Initial pH	Final pH*	Change in pH**
48 H, 37 degrees	7.013 (0.018)	6.18 (0.033)	- 0.83 (0.045)
48 H, 38 degrees	7.003 (0.015)	6.14 (0.065)	- 0.86 (0.56)
72 H, 37 degrees	7. 00 (0.026)	6.01 (0.012)	- 0.99 (0.027)
72 H, 38 degrees	7.01 (0.026)	6.06 (0.009)	- 0.95 (0.022)

* Time, $P < 0.01$; ** Time, $P < 0.02$

Figure 3.1 In vitro DMD (%) of tall fescue as affected by incubation period and temperature

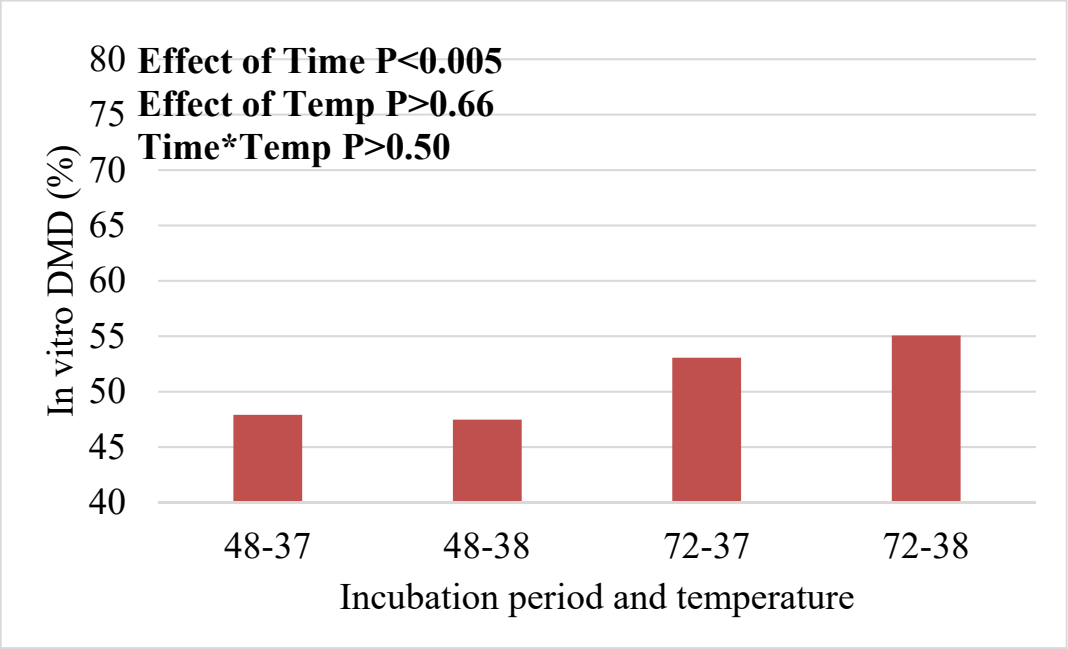


Figure 3.2. In vitro DMD (%) of timothy as affected by incubation period and temperature

75

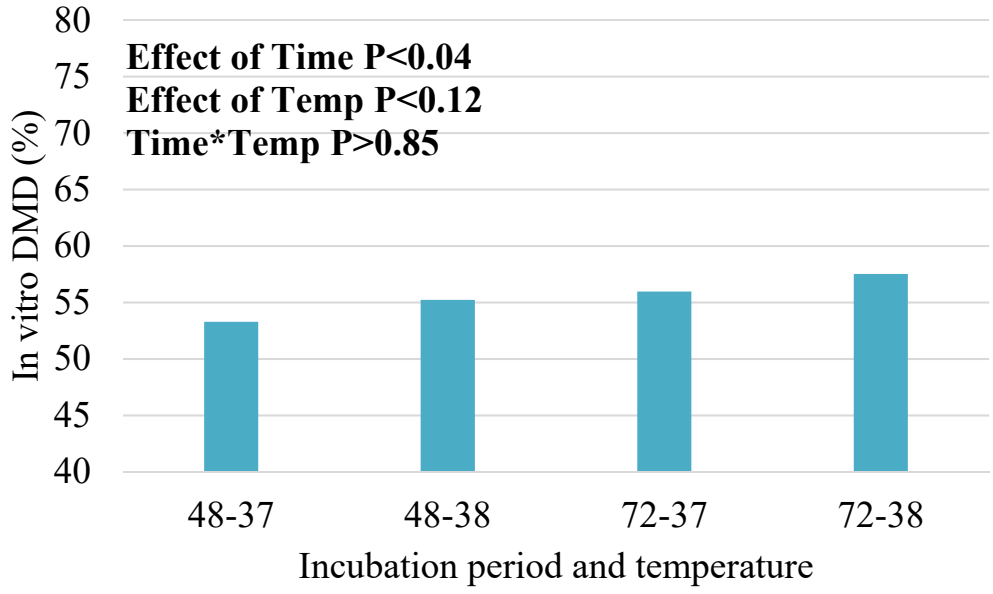


Figure 3.3 In vitro DMD (%) of alfalfa-1 as affected by incubation period and temperature

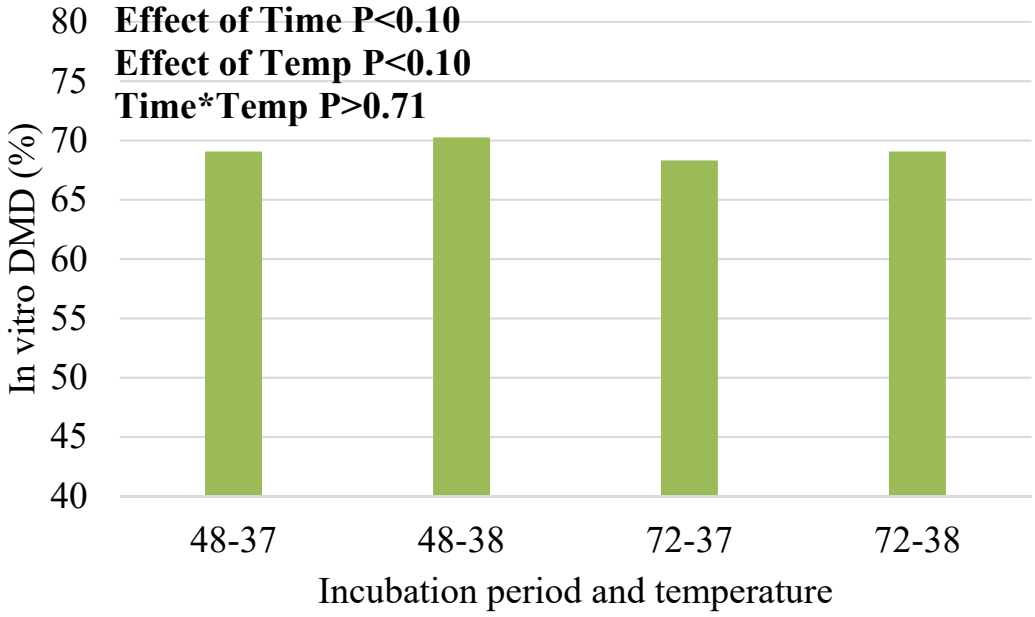
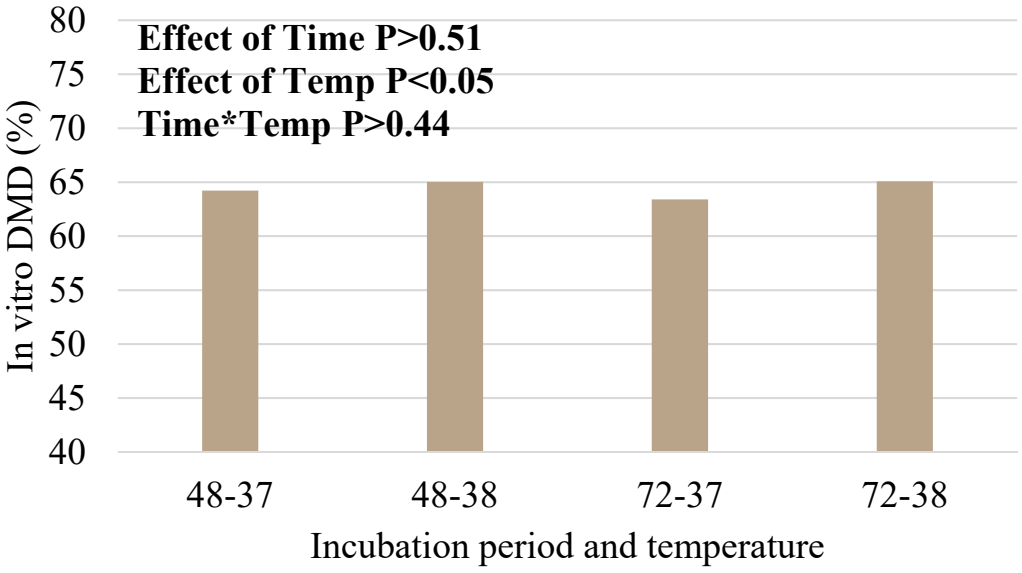


Figure 3.4 In vitro DMD (%) of alfalfa-2 as affected by incubation period and temperature



Chapter 4: Effects of forage chemical composition on dry matter digestibility in the horse determined using Daisy II

Introduction

Forages are the foundation of most equine diets, however understanding the forage quality variables on a laboratory analysis may be difficult for the average horse owner. The physical characteristics of hay may be easier for horse owners to assess, though owners may end up overpaying for hay by overvaluing some variables such as color. Failure to understand the nutritional quality of hay may cause owners to misfeed nutrients by purchasing hay that is too low in nutrient value (in the case of performance horses), or too high in nutrients (in the case of sedentary horses).

Forage quality is impacted by several variables including stage of maturity, plant type, harvest and storage conditions, some of which can affect the hygienic quality as well as the nutritional quality of the forage. Certain indices of forage quality, such as relative feed value (RFV) and relative feed quality (RFQ), have been developed from ruminant data but no RFV or RFQ indices currently exist that are equine specific. The indices used for ruminant forages were designed to link ruminant digestion and performance. Horses' digestive tracts are very different from their ruminating counterparts. Due to these differences, an equine specific index might better predict digestibility of forages by horses using forage chemical composition markers. Hansen and Lawrence (2017) surveyed published digestibility studies with horses to investigate the relationship between digestibility and forage chemical composition. The variables that were most important for predicting digestibility were neutral detergent fiber (NDF) and crude protein (CP). But the authors noted that additional data would be needed to validate their results.

Ruminant forages are sometimes evaluated using in vitro digestibility assays which are faster and less costly than in vivo digestibility determinations. Earing et al. (2010) paved the way for using in vitro methods to create a uniform means of comparing differences in forage chemical properties and dry matter digestibility by horses. The objective of this study is to evaluate the relationship between forage chemical composition and dry matter digestibility using an in vitro system with equine feces as the fermentation inoculums. In addition, these results are compared to other indicators of forage quality such as RFV and DMD predicted by composition (pDMD) according to Hansen and Lawrence (2017). We hypothesized that in vitro DMD will be affected by ADF, NDF and CP and that in vitro DMD would be correlated with other indices of forage quality including RFV and pDMD.

Materials & Methods

Forage selection and composition analysis

Thirty-one forages were selected for this experiment from a larger group of approximately 50 forages previously used in the equine nutrition program or submitted by clients for evaluation (Table 4.1). Some of the 50 forages were eliminated because the amount of available sample material was small. The remaining samples were analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein. NDF and ADF concentrations were determined using a batch system (A200 Fiber Analyzer, ANKOM Technology, Macedon NY). Crude protein was determined using an automated system (Elementar Nitrogen/Carbon Analyzer Variomax, Elementar Americas Inc, Ronkonkoma, NY).

The selected forages represented a range of NDF, ADF and CP concentrations, and included forages that were predominantly cool season grasses (n=13), predominantly alfalfa (n=11), and mixed grasses/legume hays (n=7). In the final group of samples NDF ranged from 33 to 71%, ADF ranged from 21 to 44% and CP ranged from 6.7 to 25.6% (all on dry matter basis- DMB). The compositions of the 31 forages selected for the experiment are shown in Table 4.1.

In vitro dry matter digestibility determinations

The DMD of the 31 forages was determined using 48 h and 72 h of incubation in the Daisy II at 38^{o}C. General procedures for the preparation of sample bags, buffers and inoculums were the same as described for the previous study. Based on the results of the previous experiment, only 15 sample bags were used in each Daisy II vessel. Those 15 bags contained triplicate samples of 4 different forages and 3 blanks.

Four Ankom incubation jars were used per run for a total of 60 bags per run, which allowed the simultaneous incubation of 16 forages for either a 48 or 72-h incubation period. Two in vitro runs were completed for each set of forages at each incubation period. Consequently, the DMD value for each forage was determined from the average of 3 triplicate bags within a run that was then replicated 2 times (a total of 6 observations per forage). In each run, one forage was repeated as a marker in the run that the forage was not in otherwise, to ensure DMD variation between runs was minimal (appendix 4).

Relative Feed Value and Predicted DMD

The following equation (Hansen and Lawrence, 2017) was used to predict dry matter digestibility (pDMD) from the chemical composition of each forage.

$$DMD = 65.81 + 0.7207 \times CP - 0.3514 \times NDF$$

In addition, ruminant digestible dry matter (DDM below, later referred to as rDMD), dry matter intake and relative feed value (RFV) was calculated from the following equations (Moore, 2002).

$$DMI, \%\ OF\ BW = \frac{120}{(NDF, \%\ of\ DM)}$$

$$DDM, \%\ of\ DM = 88.9 - 0.779 \times (ADF, \%\ of\ DM)$$

$$RFV = \frac{DMI \times DDM}{1.29}$$

Data management and statistical analyses

The 48 and 72 h DMD values were compared using a paired t-test. The relationships between DMD at 48 h and 72 h, and each of the chemical composition variables (NDF, ADF, and CP) were evaluated using Pearson correlation coefficients (Proc Corr, SAS, 9.4). Pearson correlation coefficients were also used to evaluate the relationship between 48 and 72 h DMD and the calculated variables pDMD, rDMD and RFV. In addition, simple linear regression (Proc Reg, SAS 9.4) was used to examine the ability of the chemical composition variables to predict 48 and 72 h DMD. Likewise, the ability of pDMD, RFV and RDMD to predict 48 and 72 h DMD was examined with simple linear regression.

Results

The 48 and 72 h DMD values and the calculated pDMD, rDMD and RFV values for all forages are shown in Table 4.2. The lowest 48 h DMD (43.09%) was observed for a grass hay (ID #51) which contained 8.3% CP, 71% NDF and 40% ADF. The highest 48 h DMD (83.66%) was observed for an alfalfa hay (ID #13) that contained 25.6% CP, 33% NDF and 21% ADF. These two forages also had the lowest and highest DMD at 72 h. Across all forages, 72 h of incubation produced higher DMD values ($P< 0.05$) compared to 48 h of incubation (64.7% and 62.7%, respectively). Figure 4.1 shows the 48 and 72 h DMD values when the forages are ranked in ascending order of digestibility. The largest differences between the 48 and 72 h values appear to occur in the least digestible forages.

Table 4.3 shows the Pearson correlation coefficients for the relationships of each variable to the 48 and 72 h DMD values. NDF ranged from 33.0 to 71.0% for the samples used in this experiment and there was a negative relationship between NDF and DMD at 48 h ($r = - 0.779$; $P < 0.0001$) and at 72 h ($r = - 0.812$; $P < 0.0001$). The ability of NDF to predict to 48 h and 72 h DMD is shown in figure 4.2. Using linear regression, NDF concentration alone, accounted for slightly more than 60% of the variation in DMD among samples (48 h R^2=0.6076; 72 h R^2=0.6591).

The ADF concentration of the forages used in this study ranged from 21.0 to 44.1% and there was an inverse relationship between ADF and the 48 h DMD ($r = - 0.826$; $P < 0.0001$) and the 72 h DMD ($r = - 0.841$; $P < 0.0001$). There was a moderately strong predictive relationship between the concentration of ADF in the forages and the 48 and 72 h DMD (Figure 4.3; 48 h R^2=0.6819; 72 h R^2=0.7072), indicating that

approximately 70% of the variation in 48 and 72 h DMD is associated with the ADF in the forage. Both the linear regression and Pearson correlation coefficients suggest that ADF had a stronger relationship to 48 and 72 h DMD than NDF concentration.

The selected forages encompassed a broad range of CP concentrations (6.7 to 25.6%) however, the relationship between CP and 48 and 72 h DMD was not as strong as for ADF or NDF. However, both 48 and 72 h DMD were positively correlated with CP concentration ($r = 0.572$; $P < 0.008$ for 48 h and $r = 0.615$; $P < 0.0002$ for 72 h). The coefficients of determination determined by linear regression for CP and 48 and 72 h DMD were (R^2=0.33 and R^2=0.38, respectively).

The predicted DMD values (pDMD) that were calculated using the equation of Hansen and Lawrence (2017) for the forages used in this experiment ranged from a low of 46.81% to a high of 72.69%, and the mean value was 57.87%. The mean pDMD was lower than mean values for the 48 and the 72 h DMD ($P < 0.05$). The rDMD values, calculated with the equation developed for ruminants, ranged from 54.62% to 72.54% and the mean value of 63.16% was intermediate to the mean values for 48 and 72 h DMD. Figures 4.5, 4.6 and 4.7 show the ability of the calculated values to predict the 48 h and 72 h DMD values for the forages used in this study. It appears that the calculated values of rDMD and RFV explain more of the variation in the 48 and 72 h DMD values than the pDMD. The slope of the regression line for pDMD versus 48 h and 72 h DMD is close to 1 but the intercepts of 4.554 (48 h) and 6.756 (72 h) suggest that the pDMD underestimates both the 48 and 72 h DMD values.

Discussion

The results of this study indicate that both incubation time and forage chemical composition affect the estimates of in vitro dry matter digestibility. The results showed an increase in forage DMD at 72 h compared to 48 h of incubation, although the difference was not large and appeared to be the greatest for the forages with the lowest digestibility.

Koller et al. (1978) performed in vitro incubations using cecal fluid from ponies for 24 and 48 h. The in vitro DMD of alfalfa hay (24.7% CP, 31.5% NDF, 23.9% ADF) had a minimal increase in DMD with increasing incubation time (81.2% to 84.5%). However, the increased incubation period had a more marked effect on the DMD of orchard grass (13% CP, 67.5% NDF, 39.4% ADF) as the 24 h DMD was 57.8% compared to 70.6% for 72 h. In this experiment the longer incubation period increased the strength of the relationships between CP, NDF and ADF and the DMD derived by in vitro analysis.

NDF concentration was found to be negatively correlated to DMD. This relationship indicates that with increasing NDF content in the forage, DMD is expected to decrease. This finding is consistent with previous studies, including Hansen and Lawrence (2017). ADF was also negatively correlated with 48 and 72 h DMD, indicating with increasing ADF content, DMD is expected to decline. This supports the NDF finding as ADF/NDF collectively makes up the fiber component of the forage. However, ADF had a stronger relationship to DMD at both 48 h and 72 h than NDF. ADF is generally considered to be less digestible than NDF, so its strong effect on in vitro digestibility is not surprising. The dry matter digestibility of forages that is calculated within the RFV equation uses ADF to predict DMD. However, Hansen and Lawrence

(2017) found that NDF had a higher predictive value for in vivo DMD than NDF for forage diets fed to horses. The reason that ADF was less predictive than NDF in their study is unknown, although their study included warm season forages in addition to cool season forages.

Crude protein concentration was not a strong predictor of 48 h or 72 h DMD, but it was positively correlated with DMD. This relationship indicates that with increasing CP content, digestibility is expected to increase. Overall, the relationships found in this study are consistent with observations on the digestibility of different forages by both horses and ruminants. Late maturity forages, which are higher in NDF and ADF and lower in CP are generally less digestible than early maturity forages that are lower in NDF, lower in ADF and higher in CP. Similarly, legumes that generally are lower in NDF and higher in CP than grasses, are usually also higher in digestibility.

Previously Hansen and Lawrence (2017) evaluated whether a model could be developed to relate forage chemical composition and forage digestibility by comparing published data from in vivo digestibility studies with horses. Although the in vivo experiments complied in the Hansen and Lawrence study may have had slightly different methods of evaluating forages, the range of forages used would provide an initial evaluation if any connections could be made between forage chemical composition and digestibility to create a model for predicting digestibility. They evaluated several regression equations and their correlation to determine which forage chemical components would best predict digestibility. The results indicated digestibility was best predicted when forage NDF and CP were included in the regression equation. In addition, Hansen and Lawrence (2017) found that NDF was the single variable most predictive of

DMD from in vivo studies using forage-only diets. In the present study, ADF was a stronger predictor of in vitro DMD. Current ruminant forage digestibility models often utilize both NDF and ADF in differing components. Relative feed value (RFV) utilizes the NDF fraction to calculate dry matter intake (DMI) whereas ADF is utilized in the digestible dry matter calculation (DDM) to produce an estimate of forage quality compared to full-bloom alfalfa (Moore and Undersander, 2002).

The results of the current study indicate a relationship between NDF and ADF and DMD, but a lesser correlation between CP and DMD. These findings had some differences from the findings of Hansen and Lawrence (2017) who previously reported that NDF was the best single variable to predict in vivo DMD. They found that the inclusion of a second variable, CP, further improved the relationship and producing the equation DMD=65.81+0.7202 x CP- 0.3514 x NDF ($P<0.0001$, $R^2=0.6690$).

This experiment also found that CP had a lower correlation with digestibility than ADF or NDF. The range of crude protein in the forages selected for use in this study was broad, 6.7 to 25.6% CP on a DM basis. Hansen and Lawrence (2017) had a slightly wider range, that included forages with CP below 6.7%. Their study also included some warm season grasses, while the only grasses includes in this study were cool season types.

Crude protein is an important consideration for use in relative feed quality equations and total digestible nutrients (TDN), both commonly used indexes for forage fed to ruminants. TDN utilizes several components of the forage including NDF, CP, neutral detergent fiber crude protein (NDFCP), non-fibrous carbohydrates (NFC), fatty acid content (FA), ether extract (EE), nitrogen free NDF, and 48 h in vitro NDF digestibility (Moore and Undersander, 2002). Furthermore, this equation differs slightly

for forage type (legumes vs grasses) and season (warm vs cool season grasses) (Moore and Undersander, 2002). A model is always limited by it data so RFQ may be ideal for predicting forage digestibility as it encompasses a wide range of data on the forage. The crude protein content of the forages used was determined using the Elementar Nitrogen/Carbon Analyzer and the variomax program to calculate percent protein. Although this method gives an approximation of protein content, it does not indicate crude protein digestibility, but is an estimate of CP of the feed based off of nitrogen content, so future studies could evaluate a relationship between digestible crude protein and DMD.

This study evaluated the relationship of an in vitro model predicting DMD for a range of forages with the hypothesis that forage dry matter digestibility would be impacted by forage chemical composition producing results similar to the findings of Hansen and Lawrence (2017). The Daisy II in vitro incubator has been proven to effectively model in vivo equine digestibility (Earing et al.,2010), but forage digestibility can differ depending on incubation period. Earing et al. (2010) found that incubation periods under 36 h were not able to produce DMD results similar to those produced in the live animal. For that reason, this study utilized two incubation periods, 48 and 72 h, to determine differences in DMD with an increase in incubation. The results showed an increase in forage DMD at 72 h compared to 48 h of incubation ($P<0.005$).

Equine fecal samples were used as an inoculum source for this method which may account for this difference. Microbes in the feces may need the additional time to reach levels similar to the microbial population in the horse, at which point forage digestion can begin, thereby resulting in differences in DMD from 48 to 72 h. The largest

difference between the 48 and 72 h DMD values appeared to occur in forages with the lowest DMD. This relationship suggests that higher quality (higher digestibility) forages are well digested by 48 h, but lower quality forages require a longer incubation period if the goal is to determine the maximum amount of digestible material in a sample. Ideally the appropriate incubation time would be the one that produces DMD values closest to in vivo. Thus, it was of interest to compare the 48 and 72 h DMD values to the pDMD that were derived from an equation developed using in vivo experiments (Hansen and Lawrence, 2017). Both incubation periods resulted in mean DMD that was higher than the pDMD. On average the 48 h DMD overestimated pDMD by about 5 percentage units and the 72 h DMD overpredicted pDMD by about 6 units. Interestingly, the mean in vitro values were quite similar to the rDMD which is based on the digestibility of forages by ruminants. However, the rDMD predicted a narrower range of digestibility (54.62 to 72.54%) than was observed in vitro at 48 h (43.09 to 83.66%), at 72 h (47.47 to 83.32%) or for the pDMD (46.41 to 72.69). These results may suggest that indices based on ruminant prediction equations will overestimate the value of low quality forage for horses.

Models are always limited by the data set so further research on this topic could include a wider range of hay samples to encompass a larger range of crude protein, NDF and ADF. A larger forage data set would be the next step to evaluate if the findings in this paper are replicable across all forages, including more legumes, cool season grasses, and warm season grasses. Other potential measures that could also be included are forage maturity at harvest and individual fiber fractions within ADF to better account for variation in relation to DMD.

Conclusion

The findings of this study indicate a relationship between forage chemical composition and dry matter digestibility. Incubation time also plays an important role in producing in vitro estimates of digestibility. This study found that ADF and NDF were negatively correlated with DMD. ADF had the strongest ability in predicting forage DMD at 72 hours, followed by ADF at 48 hours. NDF did not account for as much of the variation in DMD as ADF. Crude protein was positively correlated with DMD, but the strength of the relationship was not high. The 48 and 72 h in vitro DMD determined in this study appeared to overestimate the DMD predicted by the equation of Hansen and Lawrence (2017). Further research is needed to evaluate the relationship of chemical composition to forage digestibility and the factors influencing intake so a simple index of forage feeding value can be developed for horses.

Table 4.1. Chemical composition of the forages used Chapter 4 (dry matter basis)

Sample Title	Forage Type	ID*	CP (DM)	NDF	ADF
BD Gate 2	Legume	3	21.4	39	25
Overbrook Alfalfa	Legume	5	15.1	49	28
Forest Music	Mixed	8	12.4	56	44
OB Compressed Hay	Mixed	10	13.1	47	28
BD Alfalfa Mix	Mixed	11	17.9	42	27
Brittany Alfalfa	Legume	13	25.6	33	21
Creech Alfalfa	Legume	15	16.9	43	33
Grass Hay	Grass	17	17.8	49	31
UK-BG	Grass	18	12.8	57	32
Grass Hay Round Bales 12.5.12	Grass	21	8.6	61	31
BD Gate 2	Grass	23	10.4	63	43
BR Timothy	Grass	25	7.9	59	36
Tom's Timothy	Grass	26	8.9	51	28
Fescue	Grass	27	8.4	67	36
MC Orchardgrass	Grass	28	11.5	62	33
Alfalfa Squares High Quality	Legume	35	16.7	44	32
Mature Orchardgrass Hay	Grass	38	6.7	69	40
LL Mix	Mixed	39	16.1	56	38
Alfalfa Shattered	Legume	40	18.6	39	28
Alfalfa Leafy	Legume	41	19.3	36	27
Alfalfa Stemmy	Legume	42	17.2	44	34
Clover BG Mix	Mixed	43	17.4	52	30
Bleached Grass Hay	Grass	44	16.0	50	29
B5 Grass Timothy	Grass	48	11.8	64	35
B5 Aisle Grass Timothy	Grass	49	9.7	61	32
Main Barn Alfalfa	Legume	50	18.9	51	37
Round Bales	Grass	51	8.3	71	40
Alfalfa Mix	Mixed	52	16.2	58	30
Alfalfa Mix	Mixed	53	20.2	48	32
Farm Alfalfa	Legume	54	18.5	53	40
Mature Alfalfa	Legume	55	14.6	59	44
Mean			14.7	52.7	33.0
Range			6.7 – 25.6	33-71	21-44
SD			4.7	9.8	5.7

* Laboratory identification number

Table 4.2. In vitro 48 and 72 h dry matter digestibility, predicted dry matter digestibility (pDMD) and ruminant dry matter digestibility (rDMD) values for each forage

Forage ID	48 h DMD (%)	72 h DMD	pDMD (%)*	rDMD (%)**
3	70.93	74.92	67.54	69.43
5	73.17	76.95	59.47	67.09
8	56.73	58.98	55.06	54.62
10	74.33	75.69	58.75	67.09
11	74.02	72.54	63.93	67.87
13	83.66	83.32	72.69	72.54
15	68.60	69.43	62.87	63.19
17	64.50	66.78	61.41	64.75
18	64.85	63.02	55.03	63.97
21	61.59	60.19	50.57	64.75
23	55.53	55.79	51.16	55.40
25	57.42	56.89	50.75	60.86
26	72.63	74.72	54.32	67.09
27	52.68	54.98	48.31	60.86
28	63.37	65.53	52.30	63.19
35	60.35	65.34	62.40	63.97
38	47.08	48.54	46.41	57.74
39	55.37	57.51	57.72	59.30
40	68.32	71.68	65.51	67.09
41	69.75	72.81	67.09	67.87
42	66.50	67.60	62.76	62.41
43	68.27	68.72	60.06	65.53
44	64.73	67.50	59.68	66.20
48	66.85	69.42	51.83	61.64
49	56.19	60.11	51.38	63.97
50	61.05	62.47	61.53	60.08
51	43.09	47.47	46.82	57.74
52	54.68	59.07	57.09	65.53
53	66.15	66.56	63.53	63.97
54	59.35	62.38	60.52	57.74
55	43.27	48.84	55.57	54.62
Mean [#]	62.74[a]	64.70[b]	57.87[c]	63.16[ab]
Range	43.09- 83.66	47.47- 83.32	46.41- 72.69	54.62- 72.54
SD	9.28	8.76	6.52	4.45

* Predicted using the Equation of Hansen and Lawrence (2017)

**Predicted using the RFV equation for dry matter digestibility

[#] Means with difference superscripts are different ($P < 0.05$)

Table 4.3 The Pearson correlation coefficients for the relationships of 48 and 72 h of incubation DMD to NDF, ADF, CP, predicted DMD (pDMD), relative feed value (RFV) and ruminant DMD (rDMD)

	NDF	ADF	CP	pDMD*	RFV	rDMD**
48 h DMD r	-0.779	-0.826	0.572	0.707	0.811	0.826
P-value	<0.0001	<0.0001	0.0008	<0.0001	<0.0001	<0.0001
72 h DMD r	-0.812	-0.841	0.615	0.746	0.839	0.841
P-value	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001

* Calculated from equation of Hansen and Lawrence (2017)
** Calculated from the DDM equation in the RFV formula

Figure 4.1. Comparison of the 48 and 72 h DMD for the forages used in Chapter 4 with forages ranked from lowest to highest DMD.

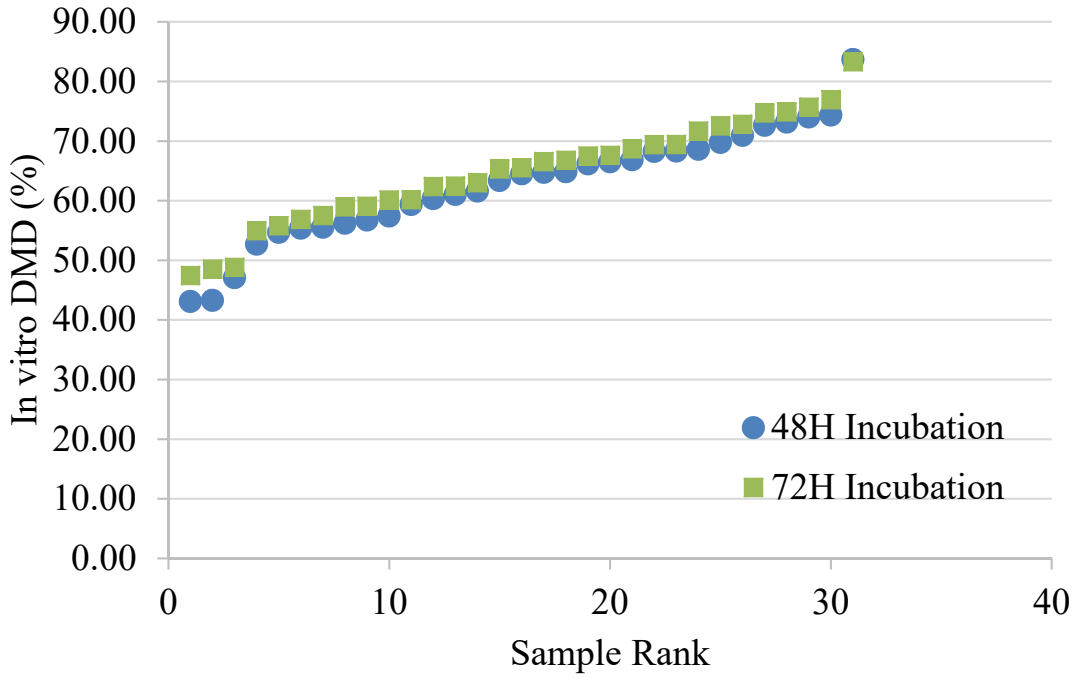


Figure 4.2. The effect of NDF concentration on 48 and 72 h DMD

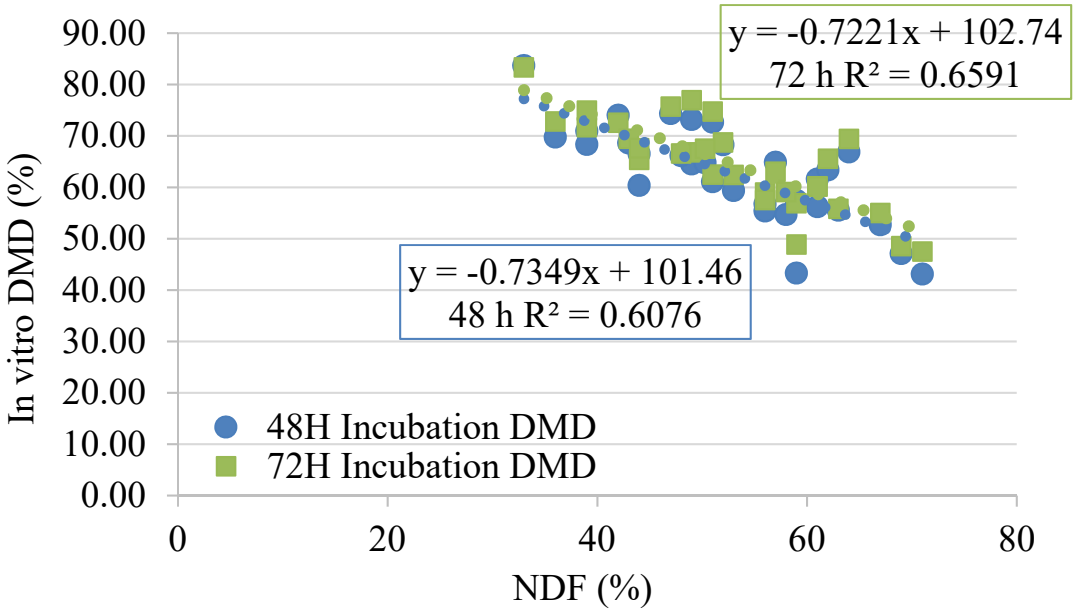


Figure 4.3 The effect of ADF concentration on the 48 and 72 h DMD

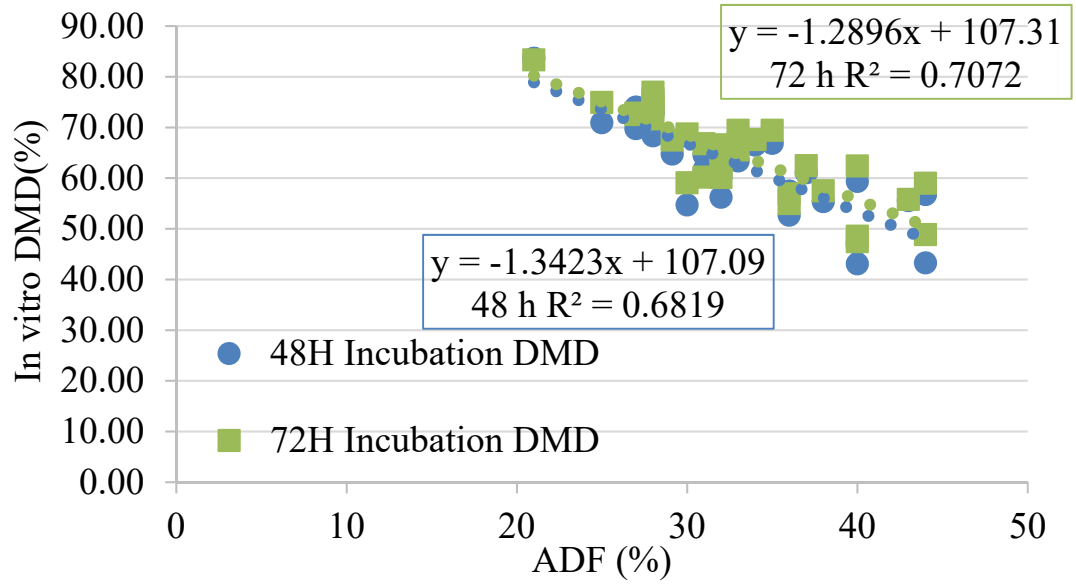


Figure 4.4. The effect of crude protein concentration on 48 and 72 h DMD

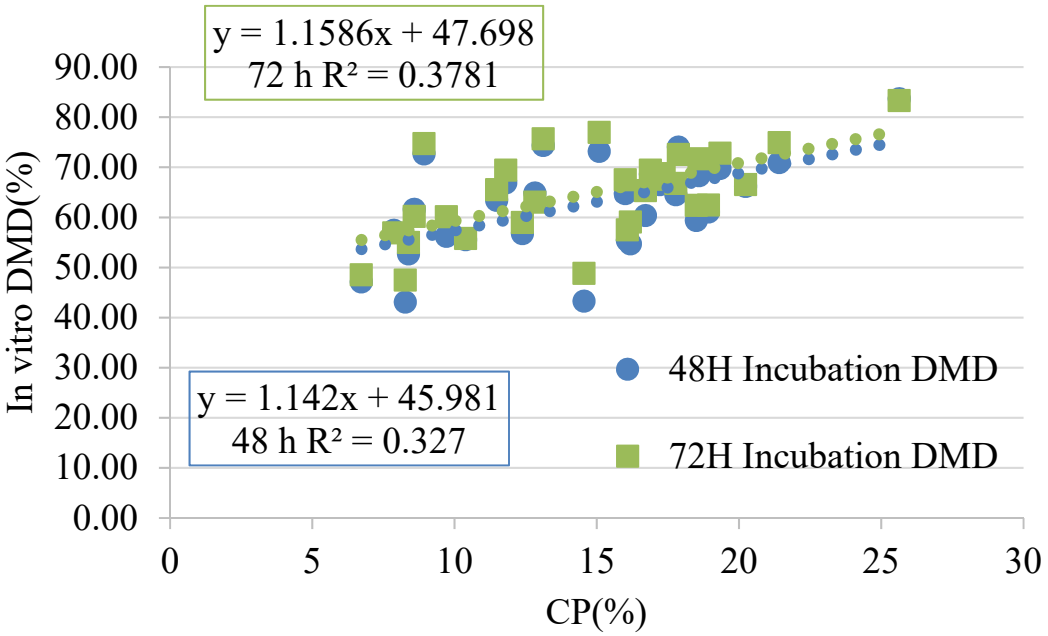


Figure 4.5 Comparison of the 48 and 72 h DMD to pDMD

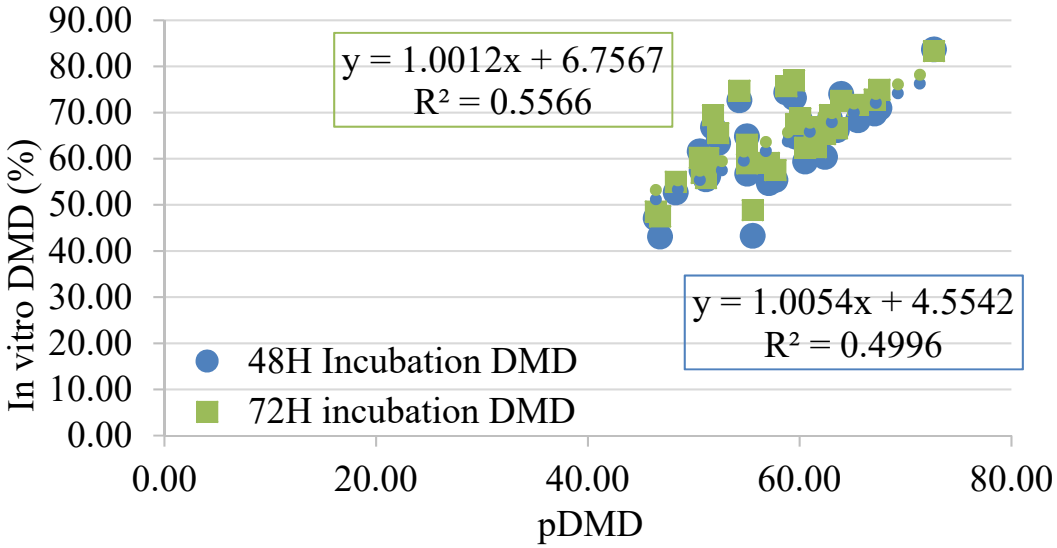


Figure 4.6 Comparison of the 48 and 72 h DMD to rDMD

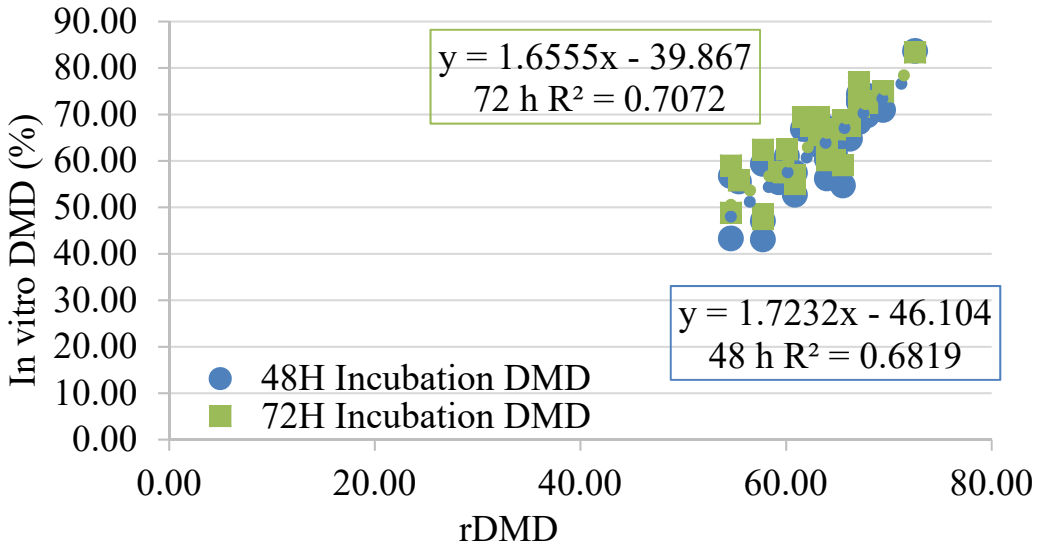
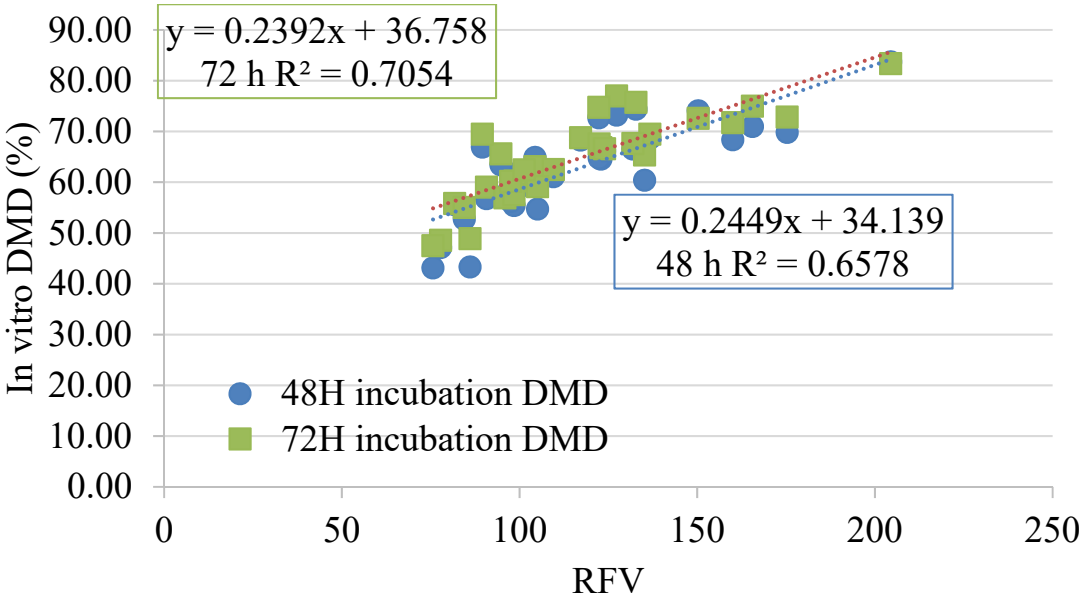


Figure 4.7 Comparison of the 48 and 72 h DMD to RFV



APPENDICES

Appendix 1- Ankom procedure for NDF Solutions for Fiber Analysis

Reagents

Neutral detergent solution	g of reagent/ 1L of distilled H_2O
sodium lauryl sulfate, USP	30.0 g
ethylenediaminetetraacetic disodium salt, dihydrate	18.61 g
Sodium tetraborate decahydrate	6.81 g
Sodium phosphate dibasic anhydrous	4.56 g
Triethylene glycol	10.0 mL

Other reagents
Alpha-amylase
Acetone

Equipment

1) Digestion apparatus- ANKOM2000/2200 Fiber Analyzer
2) Filtration device- Ankom F57 Filter bags
3) Impulse sealer to seal filter bags
4) Desiccator
5) Oven

Procedure

Sample preparation

1) Label Ankom Filter bags with black permennent marker and weigh dried bag.
2) Record tare weight and then tare scale.
3) Add approximately 0.4g of sample that has been ground to 1 mm
4) Seal sample and dry for approximately 24 hours
5) Reweigh dried sample bags.
6) Prepare three blank samples and include in digestion to determine blank bag correction.
7) Line samples into trays within Ankom apparatus.

Running NDF

1) Once samples are in Ankom apparatus, close the out-flow valve and place weight on the top of trays
2) Add 1600 ml of room temperature NDF solution to vessel (for 24 samples, Add 2000 ml)
3) Add 4 ml of heat stable alpha-amylase to solution
4) Turn on agitate and heat on (set to 100^{o}C) and confirm agitator is working
5) Set timer for 75 minutes and close apparatus so bags are sealed in vessel
6) After 75 minutes turn agitate and heat off, open the drain valve and allow solution to exit to reduce pressure within the vessel.
7) Once pressure is low, open vessel and close the drain valve. Add approximately 1600 ml of hot H_2O and 4 ml of heat stable alpha amylase. Allow rinse cycle to sit for approximately 15 minutes at 95^{o} with heat and agitation on. Repeat two times and then rinse once more without alpha amylase. Leave lid loose.
8) Remove bags from vessel and gently press out any remaining liquid from bags by pressing on bags gently with gloved fingers
9) Fill small beaker with acetone and allow bags to soak for approximately 3-5 minutes, then remove and lightly press out acetone.

10) Let bags dry out so that acetone evaporates.
11) Once dry and acetone evaporated, place bags on a drying tray and put into 105^{o} to dry overnight.
12) Once dried, remove from oven and place in desiccator while waiting for processing
13) Process samples by removing from desiccator and reweighing

Values	Calculation label
Blank Bags	
Initial tare weight	B1
Weight following NDF extraction	B2
Sample Bags	
Sample weight	WT
Sample dry weight	DM
Bag tare weight	S1
Weight following NDF extraction	S2

Calculations

Correction factor: C1= B2/B1

$$\text{NDF on a DM basis} = [52 - (51 * C1)] / [WT * DM]$$

Appendix 2- Ankom procedure for ADF Solutions for Fiber Analysis following NDF

Acid detergent solution	g of reagent added to 1 L 1.00 N H_2SO_4
Cetyl trimethylammonium bromide (CTAB)	20 g
Agitate and heat solution so that CTAB dissolves into solution	
Acetone for rinsing bags	

Equipment

1) Digestion apparatus- ANKOM2000/2200 Fiber Analyzer
2) Filtration device- Ankom F57 Filter bags
3) Desiccator
4) Oven

Procedure

1) Once samples have been run through NDF, dried and NDF calculated, take samples to run through ADF and place in Ankom vessel trays
2) Once samples are in Ankom apparatus, close the out-flow valve and place weight on the top of trays
3) Add 1600 ml of room temperature ADF solution to vessel (for 24 samples, ADD 2000 ml)
4) Turn on agitate and heat on (set to 100^{o}C) and confirm agitator is working
5) Set timer for 75 minutes and close apparatus so bags are sealed in vessel
6) After 75 minutes turn agitate and heat off, open the drain valve and allow solution to exit to reduce pressure within the vessel.
7) Once pressure is low, open vessel and close the drain valve. Add approximately 1600 ml of hot H2O. Allow rinse cycle to sit for

approximately 15 minutes at 95^{o} with heat and agitation on. Leave lid loose. Repeat three times.

8) Remove bags from vessel and gently press out any remaining liquid from bags by pressing on bags gently with gloved fingers
9) Fill a small beaker with acetone and allow bags to soak for approximately 3-5 minutes, then remove and lightly press out acetone.
10) Let bags dry out so that acetone evaporates.
11) Once dry and acetone evaporated, place bags on a drying tray and put into 105^{o} to dry overnight.
12) Once dried, remove from oven and place in desiccator while waiting for processing
13) Process samples by removing from desiccator and reweighing

Values	Calculation label
Blank Bags	
Initial tare weight	B1
Weight following ADF extraction	B3
Sample Bags	
Sample weight	WT
Sample dry weight	DM
Bag tare weight	S1
Weight following ADF extraction	S3

Calculations

Correction factor: C2= B3/B1

$$\text{NDF on a DM basis} = [53 - (51 * C2)]/[WT * DM]$$

Appendix 2- Crude Protein Procedures for Forage Protein Analysis using Elementar Nitrogen/Carbon Analyzer

1) Open Variomax computer program for collection of data and turn on Elementar machine
2) Check to ensure that gas levels (O2 and He) are appropriate and gas flow is on.
3) Dump the crucible container. Collect crucible containers and ensure they are empty and prepared for new samples
4) Weigh out approximately 500 mg of forage sample in duplicates
5) Load 2 blanks as well as 3 standards of 200-250 mg glutamic acid and repeat standard set if necessary
6) Load samples
7) Run samples (automated process)
8) Check data values to ensure there were no errors in running samples, then save data for processing

Reference

Elementar Americas, Inc. 520 Fellowship Rd. Suite D-408

Mt. Laurel, NJ 08054 USA Email: info@elementar-inc.com

Appendix 3- Ankom In vitro digestibility procedures with equine adjustments

Reagents

Buffer solution A	g/L of A reagents	Buffer solution B	g/L of B reagents
KH_2PO_4	10	Na_2CO_3	15
$MgSO_4$* $7H_2O$	0.5	Na_2S*$9H_20$	1
NaCl	0.5		
CaCl*$2H_20$	0.1		
Urea (reagent grade)	0.5		

Equipment

1) DAISYII Incubator system
2) F57 Filter Bags
3) 1915/1920 Heat Sealer/Impulse bag sealer
4) 4 DAISY II Incubation vessels
5) Water bath
6) 4 mason jars

Procedure

<u>Pre-incubation set up (72+ hours before start of experiment)</u>

1) Soak Ankom F57 filter bags in acetone for approximately 5 minutes then remove and allow to dry.
2) Once dry filter bags can be labeled with a black sharpie and dried in an oven overnight to acquire dry bag tare weight.
3) Remove samples after baking and record tare weight.

4) Sample can then be added to filter bags, sealed, and dried to determine sample dry

Incubation set-up using equine feces as inoculum source

1) Prior to arrival of equine feces turn on DAISY II incubator and water bath. Place mason jars in water bath to pre-warm and ensure all apparatuses are pre-warmed for arrival of fecal sample.
2) Mix 1300 mL of buffer A with 500 ml of buffer B for each incubation jar
3) Prior to the addition of fiber bags adjust pH of buffer A+B to approximately 7.0 using the addition of buffer A(acidic) and buffer B(basic)
4) Once solution is approximately neutral add F57 samples
5) Incubate jars in DAISY II incubator prior to arrival of equine feces to keep solution close to 37^{o}C
6) Upon arrival of feces mix the sample thoroughly and then weigh 200 g of feces into each of the mason blender jars and then flush with CO_2 and cap to await further processing. Keep jars in water bath while awaiting processing to help keep feces warm.
7) Once all fecal samples have been weighed out process one at a time by taking the blending jar and adding approximately 400 ml of buffer solution from the recipient incubator jar.
8) Re-flush with CO_2 for approximately 20 s, cap with blending cap and blend for approximately 30 s or until thoroughly mixed
9) Pour fecal slurry into respective incubator jar, mixing gently, and then take ph. If ph is not within 6.8-7.2, adjust pH to within this range by adding additional buffers (A or B as needed)
10) Once neutral ph has been reached flush with CO_2 for 30 seconds, cap tightly and return jar to Daisy incubator
11) Record time and start timer
12) Repeat for each jar
13) Leave jars to incubate for designated incubation period

Post incubation procedures

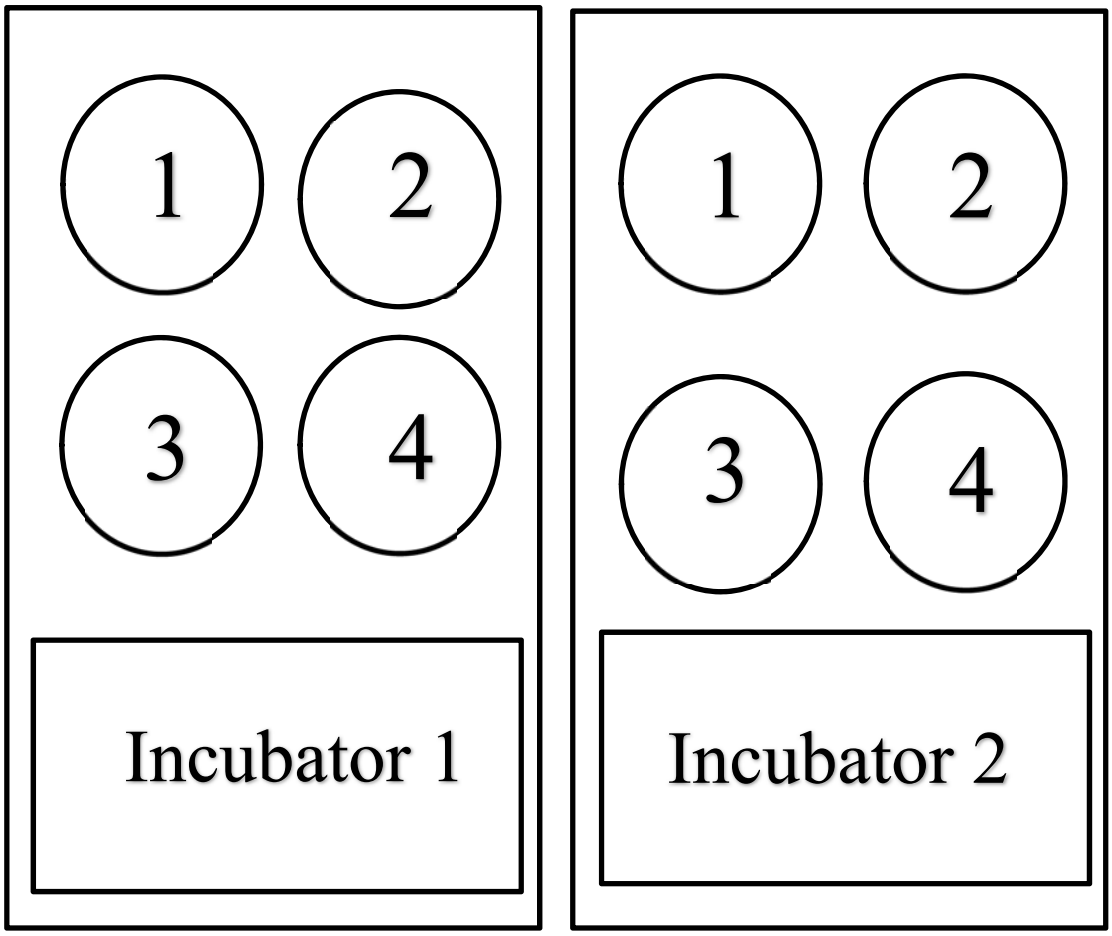
1) Once incubation period is over remove incubation jars one at a time and process individually
2) Record pH post- incubation
3) Empty contents into a colander to retrieve samples gently rinsing each one with cold water.
4) Lay forage bags over a screen, rising each side of individual bags until water runs clear and fecal residue is removed
5) Once forage bags are clean, lay flat over metal oven container and place in over at approximately 100° oven to dry overnight
6) Once samples are dried, remove to desiccator for reweighing and calculating DMD

Calculate in vitro dry matter digestibility using the following equation:

$$DMD\ (\%) = 100 - (\frac{(post\ DMD\ dry\ wt - filter\ bag\ tare\ wt \times CF)}{sample\ and\ filter\ bag\ dry\ wt}) \times 100$$

Note: Because samples were weighed dried, results of the calculation will already be on a DM basis.

Appendix 4- Ankom DAISY II incubator set up visual



Forage separation was conducted as shown below.

Run 1 (a, b)
Forages 1-4 vessel 1
Forages 5-8 vessel 2
Forages 9-12 vessel 3
Forages 13-16 vessel 4

Run 2 (a)
Forages 17-20 vessel 1
Forages 21-24 vessel 2
Forages 25-28 vessel 3
Forages 29-31+Marker vessel 4

a- For run 3+ 4 the incubators were switched and the positions of forages in the vessels were different.

b- One forage in run 1 was repeated in run 2 as the “marker” forage.

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Vita

Veronica Bill was born in Williamsburg, VA and grew up just outside of Annapolis, MD. She graduated from the University of Kentucky College of Agriculture, Food and the Environment with a Bachelor of Science in Equine Science and Management, and a dual minor Agriculture Economics and Animal Science. As an undergraduate, Veronica worked on several equine and forage related research studies within the College of Agriculture, Food and the Environment, within both the Animal Science department and Department of Plant and Soil Science.

During her undergraduate career, Veronica served as an Agriculture Ambassador for the college and taught as an undergraduate teaching assistant in the Agriculture Economics department and was a guest lecturer in the Plant and Soil Science department. Her junior research project, which evaluated the effect of plant growth regulators and nitrogen in red clover growth and partitioning, placed 3rd at the 2014 ASA, CSA, SSSA International Meeting in Research Symposium Oral Presentations. She also helped the UK forage team place 1st at the AFGC national forage bowl in 2014, and served as an undergraduate team leader for the forage bowl team in 2015.

In the fall of 2015, Veronica began her Master of Animal Science under the direction of Dr. Laurie Lawrence. Her primary research focus was looking at forage chemical composition and the effect of digestibility in the horse through in vitro methods. In addition to her master's research project, Veronica conducted research evaluating a proprietary product for use in horses. Veronica served as a teaching assistant for an undergraduate animal nutrition class and

guest lectured for several college classes. Her interest in cooperative extension also led Veronica to volunteer at several equine extension events throughout her time in graduate school.

Publications and Abstracts

Pyles, M.B., A.L. Fowler, V. Bill, B.E. Harlow, A. Crum, S.H. Hayes, M.D. Flythe, and L.M. Lawrence. 2016. Age-related changes in select fecal bacteria in foals. American Society of Animal Science Meeting. [Abstract #18192] (Oral)

Pyles, M.B., A.L. Fowler, V. Bill, B.E. Harlow, A. Crum, S.H. Hayes, M.D. Flythe, and L.M. Lawrence. 2016. Effect of starch source in pelleted concentrates on fecal bacterial communities in Thoroughbred mares American Society of Animal Science Meeting. [Abstract #18128] (Poster)

Bill, V., B. Goff, L. Harris, S. R. Smith, B. Hadley. 2014. The effect of plant growth regulators and nitrogen in red clover growth and partitioning. Abs. Proc. ASA, CSA, SSA International Meeting. Long Beach, CA. 19-9 (oral)

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