

Changes in Fecal Microbial Populations in Horses
Maintained on Various Diets

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Abstract

Laminitis, a condition of the hoof, is one of the most common and devastating conditions that affects the horse (*Equus caballus*). The most common factors responsible for triggering laminitis are intake of excess grain or exposure to lush pastures where high starch rich diets are consumed, although the connection between the gut and the hoof is not well understood. When horses are fed starch rich diets, like grain commonly found in the diets of race and performance horses, the balance of microbial species present in the digestive tract of the horse become disturbed, leading to lactic acidosis in the horse. The objective of this study was to identify and quantify the fecal microbial populations in horses maintained on various diets. Microbial populations present in the equine hindgut were assayed by using bacterial ribosomal DNA fragments present in fecal samples. Identification and quantification of specific bacterial species, using bacterial primers and florescent probes, can be detected by quantitative real-time PCR (qPCR). This study focused on equine hindgut streptococcal species (EHSS), including *Streptococcus lutetiensis*, which accounts for approximately 70% of the microbiota present in the hindgut prior to the onset of laminitis. Our results suggest that avoidance of pasture for all laminitic prone horses may need to be reevaluated as the hindgut microbe sensitivity to diet is unique for each individual horse. The use of these assays will be valuable in future work exploring the changing microbial populations present in the equine hindgut.

Key words: horse, laminitis, gut microbiome, hindgut fermentation, equine hindgut streptococcal species (EHSS)

Introduction

Laminitis is a devastating condition that affects the foot, or hoof, of the horse (*Equus caballus*). Laminitis is characterized by swelling and major damage to the basement membrane within the hoof and eventual separation of the dermis and underlying bone from the epidermis and hoof horn at the lamellar dermal-epidermal junction (Milinovich et al., 2008). It can affect a single hoof or all four hooves simultaneously and current available treatments are only able to alleviate the symptoms.

The most common factors responsible for laminitis are intake of excess grain or exposure to pastures where high starch rich diets are consumed (Milinovich et al., 2008). Fermentation of carbohydrates present in the forage is carried out by microbial enzymes present in the cecum and colon of the horse. This fermentation produces volatile fatty acids which are easily utilized by the horse as an energy source. However, when horses are fed starch rich diets, like grains commonly used in the diets of race and performance horses, the starch is not well utilized as an energy source. Much of the starch passes through the small intestine without being absorbed and enters the large intestine, where it is fermented by rapidly adapting hindgut microbial populations (Milinovich et al., 2006). Rapid fermentation causes a dramatic increase in the production of volatile fatty acids and lactate, leading to a subsequent drop in pH level. This acidic environment is now a perfect area for the rapid development of lactic acid producing bacteria. The development of lactic acid producing bacteria further increases total lactic acid production and decline in pH. The cycle continues as the large intestine of the horse enters a state of lactic acidosis. This lactic acidosis often precedes the development of laminitis, although how this disruption of the gut leads to damage in the foot is not yet

clear (Al Jassim et al., 2005). Potential initiators of laminitis associated with this disruption of the hindgut microbes are believed to include lamellar ischemia resulting from hindgut derived endotoxins (Garner et al., 1978; Moore et al., 1979; Sprouse et al., 1987; Zerpa et al., 2005), vasoactive agents like amines and histamines (Bailey et al., 2002; 2003; Garner et al., 2002), or uncontrolled activation of host matrix metalloproteinases (used in growth and remodeling of hoof wall) (Pollitt, 1996; Kyaw-Tanner et al., 2004).

Obesity and metabolic syndrome have been linked to the development of laminitis. Elevated insulin and triglycerides are cardinal signs of a pre-laminitic, equine metabolic syndrome (Kronfeld 2005), and a recent study demonstrated that hyperinsulinemia alone could initiate laminitis (Asplin et al., 2007). Obesity is associated with insulin insensitivity and this insensitivity is one of the characteristic and common features that link various metabolic disorders. These links are important as it was recently found that 51% of horses examined were determined to be overweight or obese, and could be subject to serious health problems like laminitis and hyperinsulinemia as a result (Thatcher et al., 2007).

A United States Department of Agriculture (USDA) survey (2000) found that laminitis accounts for up to 15 percent of all lameness problems occurring in horses. Of the total number of horses diagnosed with laminitis, approximately 4.7 percent die or must be euthanized. Overall, fifty percent of laminitis cases were determined to be caused by overgrazing in lush pastures and feeding of high starch diets (USDA, 2000). Current treatments can only address symptoms and pain, which can be debilitating. Little can be done to reverse the damage within the hoof. Therefore, when a horse is diagnosed

with laminitis, treatment can be very expensive and burden the owner with tough decisions as to how to balance quality of life for the animal and their own economic pressures. However, with a better understanding of what constitutes the environment within the equine large intestine during periods of lactic acidosis and laminitis, preventative guidelines and better, less expensive treatment options may be developed. These guidelines and treatment options will benefit 4.6 million Americans in the horse industry found at private farms, research institutions, racetracks and many other venues (American Horse Council Foundation, 2005). This research is imperative in making strides to reducing the number of horses diagnosed with laminitis every year.

The objective of this study was to measure fecal microbial populations in horses maintained on various diets. While previous studies have identified and quantified some bacterial populations in the equine hindgut, they have done so only after specific experimental conditions that can induce laminitis, like administration of large doses of oligofructose (Milinovich et al., 2008; Al Jassim et al., 2005). During this study the experimental conditions and the equine hindgut microbial community that were examined, was not induced by the researchers, but instead represented common management strategies found in industry. Bacterial species that were of great importance to this study included equine hindgut streptococcal species (EHSS), specifically *Streptococcus lutetiensis*, which has been found to account for approximately 70% of the microbiota present in the hindgut prior to the onset of laminitis (Milinovich et al., 2008). We used horse fecal matter, which can be collected using much safer and non-invasive methods than cecal fluid collected through a fistula, to examine the microbial populations present in the equine hindgut.

Teff Hay Study

Materials and Methods

This experiment was conducted at The Pennsylvania State University (PSU) as part of a multi-institution collaboration with Dr. W. Burt Staniar, and as part of an ongoing dietary study with the PSU resident Quarter Horse herd. The animal protocols for this study were approved by the PSU Institutional Animal Care and Use Committee.

Animals and Diets

Housing for the six nonpregnant American Quarter Horse mares used in this study was at the John O. Almquist Research Center in University Park, PA, during January and February of 2009. The horses had access to fresh, clean water and salt blocks (containing only NaCl) for the duration of the experiment.

Testing of three different maturities of teff hay (*Eragrostis tef*) (designated as boot, early-heading and late-heading maturities) revealed different nutrient compositions (Table 1). The study began with eight days for the horses to acclimate to experimental conditions and the research center. During this time, the horses received twice daily feedings of a hay mix consisting of equal parts of each maturity. Following the acclimation period, three successive periods lasting 12 days each occurred to allow for adaptation to the new diet. The overall study included three teff maturities, three periods and six horses used in a replicated balanced Latin square design. During each period, the mares received the experimental diet twice daily (0700 and 1900 hour, Eastern Standard Time).

Table 1: Nutrient Composition of boot, early-heading and late-heading maturities of teff hay (Adapted from Staniar et al., 2010)

| Item | Boot | Early-Heading | Late-Heading |
|-----------|------|---------------|--------------|
| DM, % | 92 | 92.1 | 92.5 |
| CP, % | 16.4 | 10.8 | 7.5 |
| ADF, % | 35.7 | 40.2 | 41.5 |
| NDF, % | 68.1 | 71.1 | 70.8 |
| Lignin, % | 3.6 | 4.0 | 4.0 |

Sample Collection

Collection of fecal samples from each horse occurred during the three final days of each 12 day period. The researchers collected samples three times daily (0700, 1200 and 1900 hour, Eastern Standard Time) from the most recently deposited fecal piles. Storage of samples in individually labeled 50 ml conical vials occurred at -80°C until ready for processing.

DNA extraction

Extraction of bacterial DNA from all fecal samples occurred using the Stool Pathogen Protocol from the QIAamp DNA stool mini kit (Qiagen, Mississauga, Ontario, Canada) with several modifications. We combined 10 ml Buffer ASL™ with 4 g of fecal sample in a 50 ml conical vial and then vortexed for 60 seconds on the highest setting. We homogenized each sample using the Polytron PT 2100 (Kinematica, Bohemia, NY, USA) on the lowest setting for 10 seconds. Following homogenization, the material was filtered through a strainer (pore size=0.9mm x 0.9mm) into a new 50 ml conical vial. Two ml of this filtered lysate was transferred into a 2 ml microcentrifuge tube. At this point we followed the published protocol at step 3, with the exception of heating the suspension at 95°C instead of 70°C. Finally, additional modifications included using 400

μl Buffer ALTM (in step 11) and 400 μl ethanol (in step 13). DNA concentration was obtained using an AlphaSpec Spectrophotometer at 260 nm (Alpha Innotech, San Leandro, CA, USA). For quantitative polymerase chain reaction (qPCR), DNAs were diluted to 25 ng/ μl using Nuclease Free Water (Qiagen, Mississauga, Ontario, Canada).

Quantitative real-time PCR

We amplified bacterial 16S rRNA genes through qPCR using a Mastercycler ep Realplex (Eppendorf, Westbury, NY, USA). Primers and probes (IDT, Coralville, IA, USA) used for species identification were previously described for all bacteria (“AllBac”) (Nadkarni et al., 2002) and equine hindgut streptococcal species (“EHSS”) targets (Klieve et al., 2003). We used KAPA Probe Fast Universal 2X qPCR Master Mix in all reactions according to the manufacturer’s recommended conditions (KapaBiosystems, Woburn, MA, USA). The primer to probe ratio used in all reactions was 5 μM primer:500 nM probe. qPCR reactions contained 20 μl total volume with 1 μl of DNA extracted from fecal samples, diluted to 25 ng/ μl . We included 2 μl of Bovine Serum Albumin (New England Biolabs, Ipswich, MA, USA) at a 1 $\mu\text{g}/\mu\text{l}$ concentration and Nuclease Free Water in each assay. We modified the thermocycle parameters for this assay to the following: initial denaturation at 95°C for 2 minutes, then 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Statistical Analysis

We evaluated the data generated from qPCR using the LinReg PCR program in order to estimate the efficiency value for each sample amplification curve (Ramakers et al., 2003). Assays were excluded if their calculated efficiencies exceeded two standard

deviations from the mean or if the R^2 values for the linear fit estimating efficiency were below 0.975. When possible the assay was repeated and reincluded if it met these criteria for reaction efficiency on a second attempt. The relative expression of EHSS was calculated based on mean reaction efficiency and relative to the AllBac reference gene (Scheffe et al., 2006). We tested for significance and designed graphs using the JMP 8.0 Statistical Discovery Software (SAS Institute, Cary, NC, USA).

Results

Hay Maturity

The detected proportion of EHSS (%EHSS) changed significantly between the boot and late-heading maturities of teff hay (student t-test, $p=0.0129$). There was no significant difference between the boot and early-heading maturities, or the early-heading and late-heading maturities (student t-test, $p=0.4057$ and $p=0.0955$, respectively) (Figure 1). Comparisons of the 3 maturities within individual horses by ANOVA revealed five out of the six horses had no significant differences in %EHSS among the three maturities of teff hay. However, %EHSS for one horse (Horse 6) was different between the boot and late-heading maturities (consistent with overall finding), and between early and late-heading maturities (student t-test, $p=0.0001$ and $p=0.0150$, respectively).

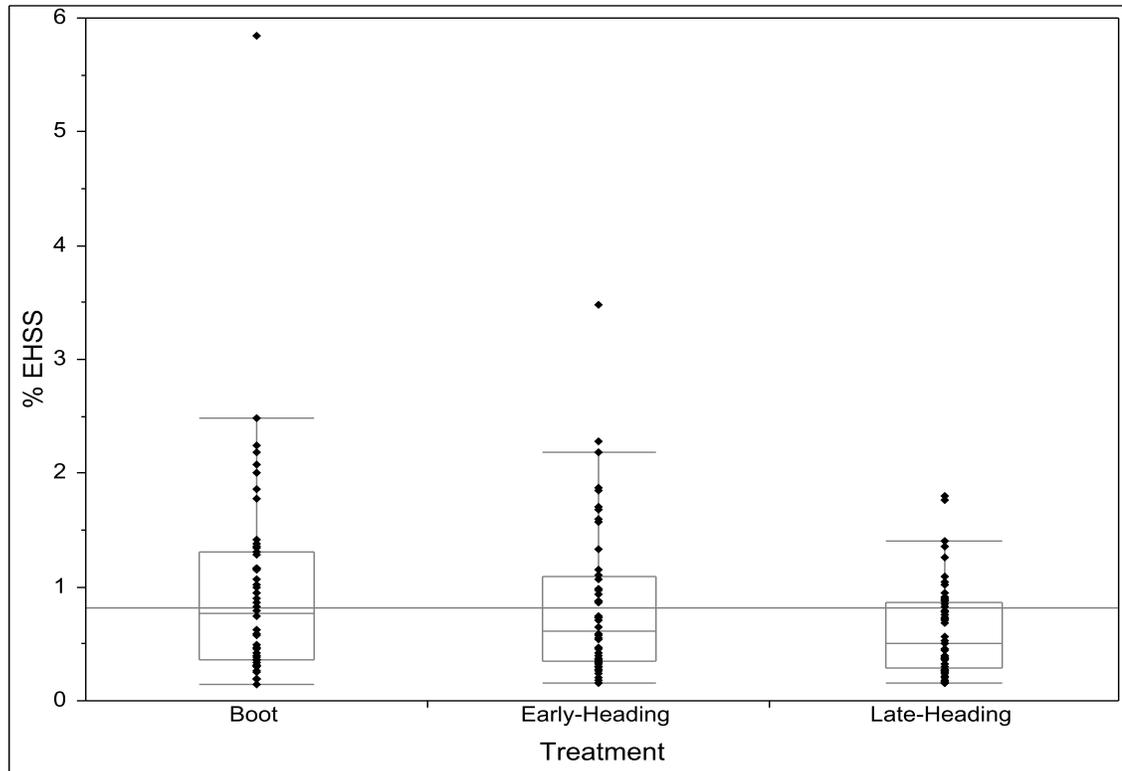


Figure 1: %EHSS (EHSS relative to AllBac) in all horses (n=6) at all time points under different maturities of teff hay. Box plot displays are median and quartile values.

Days

There was no significant difference in %EHSS between the three days of the experimental protocol.

Time

Among all horses there was a statistically significant difference in %EHSS between the 0700 and 1200 hour (student t-test, $p < 0.0001$), and the 1200 and 1900 hour (student t-test, $p = 0.0007$) sampling (Figure 2). Individually, Horses 1 and 3 each showed significant differences in the 0700 and 1200 hour, and 1200 and 1900 hour times (which

was consistent with overall findings). However, within each of the other 4 horses there were no significant differences at all three sampling times.

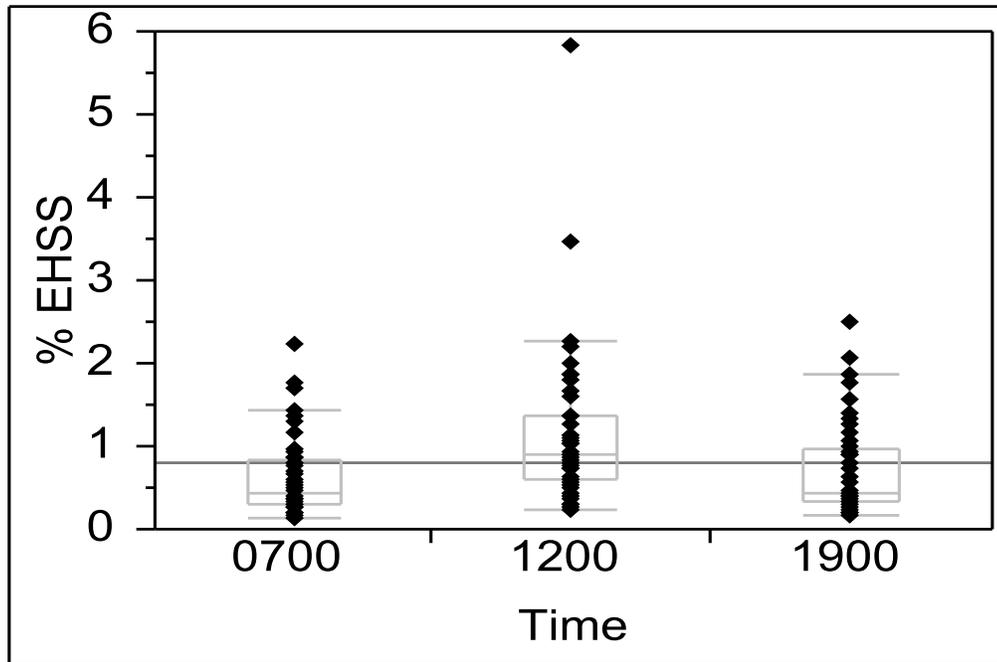


Figure 2: %EHSS (EHSS relative to AllBac) in all horses (n=6) at three different fecal collection times. Box plot displays are median and quartile values.

Miner Institute Study

Materials and Methods

The animal protocols for this study were approved by William H. Miner Agricultural Research Institute Animal Care and Use Committee.

Animals and Diets

Housing for the eight Morgan horses used in this study was at the William H. Miner Agricultural Research Institute, during June to August of 2009. Assignment of the eight horses to two treatment groups of equal size (n=4) was based on age, sex, bodyweight and body condition score. The horses received a low nonstructural carbohydrate (NSC) mix of grass hay and alfalfa to extend the feed source over the course of the study, providing a consistent diet.

The first two weeks of the study served as a baseline, with all horses housed in box stalls and fed a hay diet which was low in NSC, at a rate of 2.0 % of their body weight. During the second 2-week period, one group of horses had access to pasture, while the second group of horses remained in box stalls consuming the low NSC hay. The third 2-week period served as a washout period to reduce any carryover effect of dietary treatments, with all horses being housed in box stalls receiving the low NSC hay. During the final 2-week period, the group previously consuming the low NSC hay in box stalls had access to pasture, while the pastured group remained in the box stalls consuming the low NSC hay. Evaluations of nutrient compositions of experimental diets are pending.

Sample Collection

Collection of fecal samples from each horse took place on the first day of the study. Within each two week period, collection of fecal samples from each horse occurred three hours post-feeding on days 3, 7, 10 and 14 of the period. The animal caretakers extracted three to five balls of feces directly from the rectum by palpation.

DNA Extraction

DNA extraction proceeded as previously reported with one additional step. We subjected the eluted DNA from the QIAGEN protocol to the Genomic DNA Clean and Concentrator protocol (Zymo Research, Irvine, CA, USA). We used 5 volumes of ChIP DNA Binding Buffer in Step 1, and the protocol was modified in step 5, to elute the DNA in 40 μ l of 65°C TE buffer. Prior to qPCR, we determined the DNA concentrations and diluted the samples to 25 ng/ μ l.

Quantitative real-time PCR

Amplification of bacterial 16S rRNA genes occurred as above. We modified the thermocycle parameters for this assay to the following: an initial denaturation at 95°C for 2 minutes, then 40 cycles of 95°C for 3 seconds and 65°C for 30 seconds.

Statistical Analysis

Data analysis was identical to the above described procedure.

Results

Diet

The EHSS population, relative to the total bacterial population (%EHSS), changed significantly between the hay mix and pasture diets (student t-test, $p=0.0018$), the hay mix and grass hay during the first period (student t-test, $p=0.0227$) and the pasture and grass hay during the second period (student t-test, $p=0.0203$) (Figure 3).

Comparisons of the dietary protocols within individual horses by ANOVA revealed, four out of the eight horses (horses B, C, D and G) had no significant differences in %EHSS between the diets. However, horses A, E and F had variations in %EHSS between the hay mix and grass hay during the 2nd two weeks (student t-test, $p=0.0458$, $p=0.0297$, and $p=0.0255$, respectively). Additionally, horses E and H had differences between the hay mix and pasture diets (student t-test, $p=0.0316$ and $p=0.0186$, respectively).

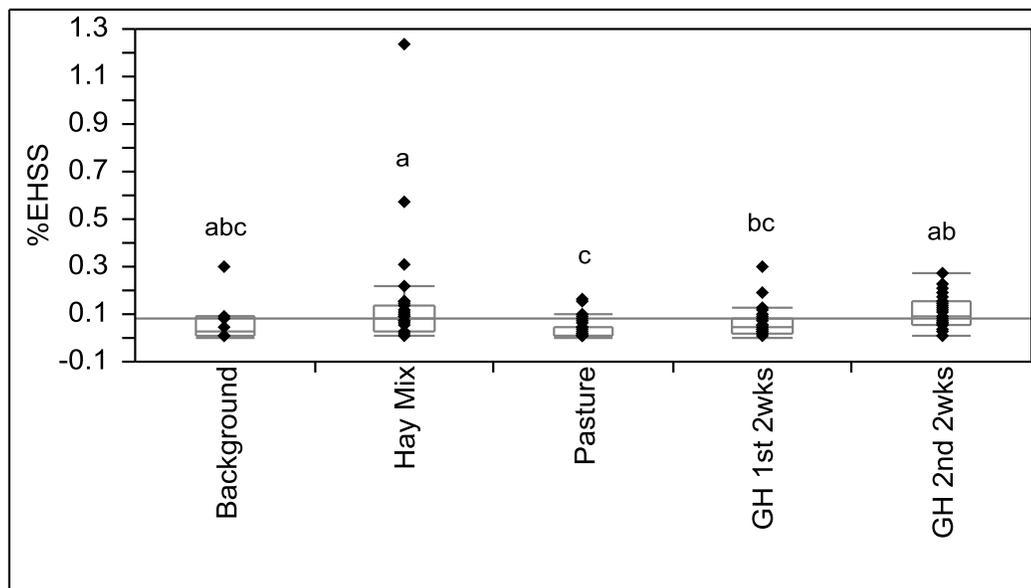


Figure 3: %EHSS (EHSS relative to AllBac) in all horses (n=8) under different dietary treatments. Different letters indicate significant differences between treatments with $p<0.05$ (student t-test). Box plot displays are median and quartile values. (GH=Grass Hay)

Day

There was no significant difference in %EHSS between day one of the experiment and days 3, 7, 10 and 14 of each two week dietary protocol.

Treatment and Day

Thirteen out of the 17 treatment and day periods (represented with a designation of bc) did not have significant changes in EHSS populations (Figure 4). Pasture day 14 had the lowest %EHSS mean value of 0.011, whereas hay mix day 7 had the highest %EHSS mean value of 0.233, which represented a statistically significant difference (student t-test, $p=0.0005$).

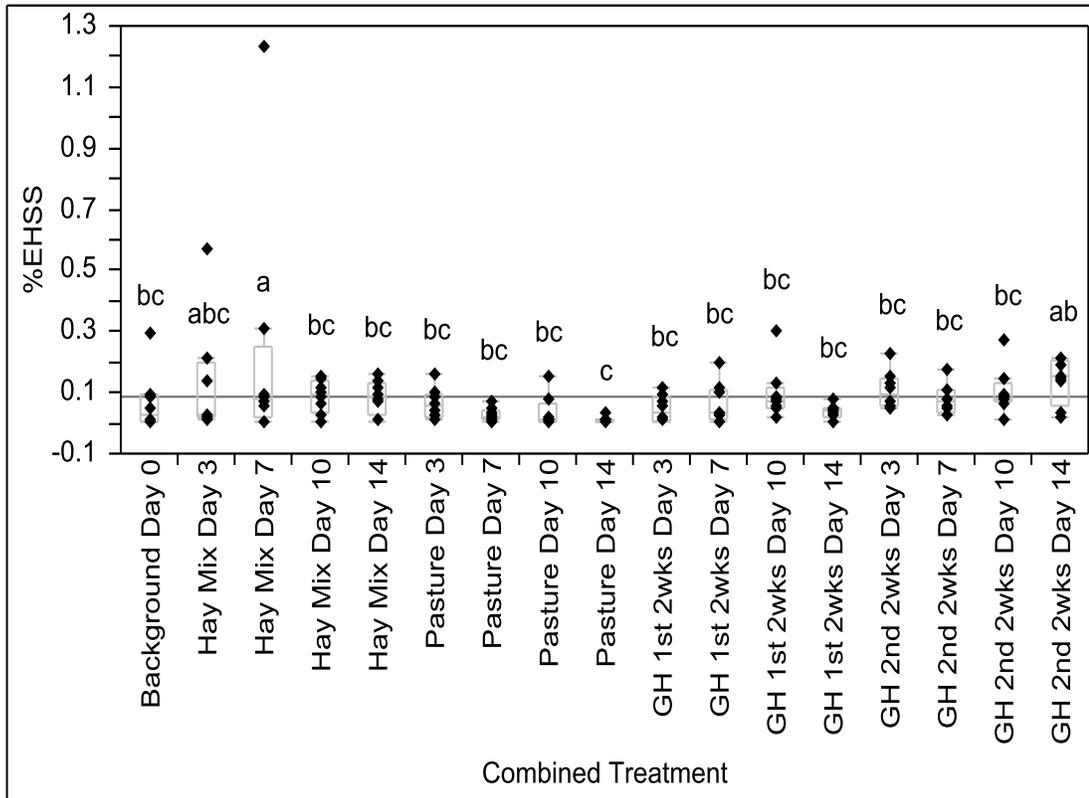


Figure 4: %EHSS (EHSS relative to AllBac) during each diet and day. Different letters indicate significant differences between treatments with $p < 0.05$ (student t-test). Box plot displays are median and quartile values. (GH=Grass Hay)

Individual Horses

Comparison of all days and diets for each horse by ANOVA revealed that the %EHSS for horse C was statistically significant from horses A, D, F, G, and H (student t-test, all $p < 0.05$). Additionally, the %EHSS for horse B was significantly different from horses A, F, and H (student t-test, $p = 0.0077$, $p = 0.0151$ and $p = 0.0221$, respectively) (Figure 5).

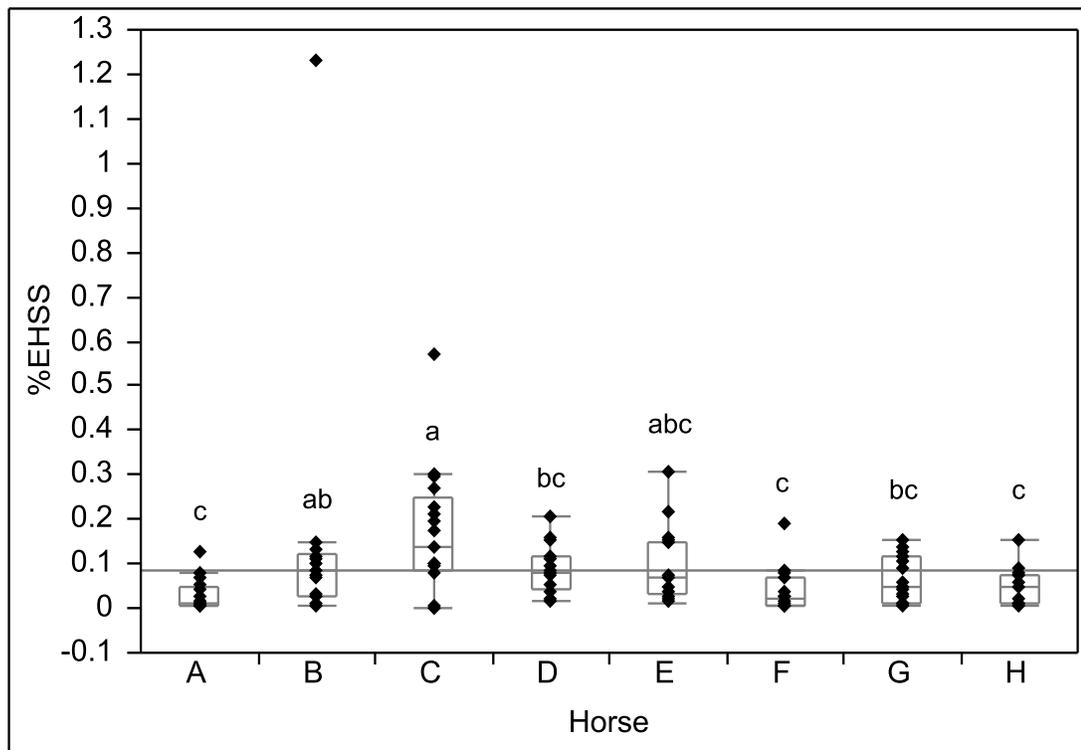


Figure 5: %EHSS (EHSS relative to AllBac) in each horse (n=8) during all diets and days. Different letters indicate significant differences between treatments with $p < 0.05$ (student t-test). Box plot displays are median and quartile values.

Discussion

Although many factors contribute to initiating laminitis, in previous studies using experimental models of induction EHSS was identified as the most probable etiological agent of equine laminitis due to colitis, or swelling of the large intestine. Milinovich et al. suggested that EHSS bacteria lyse and the released cellular components may trigger a factor in initiating laminitis (Milinovich et al., 2006, 2007). Although this process is not well understood, the role of EHSS in the development of laminitis is of extreme concern for all horse owners.

Teff Hay Study

Teff hay has a reduced nonstructural carbohydrate (NSC) content, decreasing its digestible energy (DE) content and making it a potentially beneficial feed for horses that are insulin resistance and have an increased risk of laminitis. However, in the concurrent intake and digestibility study, all horses voluntarily consumed 15% less late-heading teff hay and it was less digestible compared to the boot maturity. The recommended National Research Council (2007) requirements for DE and crude protein (CP) were not met due to this reduced intake of late-heading teff hay (NRC, 2007). Therefore, these results suggest that the late-heading maturity of teff hay should not be recommended for laminitic prone horses due to risk for colic, low palatability and reduced nutritive value (Stanjar et al., 2010). Measurements of the hindgut microbial population indicate that the late-heading teff hay has a lower mean proportion of EHSS than the earliest maturity (boot) in all horses. However, the late-heading teff hay did not have the lowest mean EHSS population amount for all individual horses. Therefore, although this hay seems

like a promising diet for laminitic horses, individual horses may respond differently and the diet should still be carefully evaluated. For five out of six horses in this study, no significant difference in EHSS populations was found between the hay maturities. Yet, one horse had significant EHSS population differences between the three maturities of teff hay. These findings are consistent with previous work documenting differing EHSS populations between individual horses after oligofructose dosing (Milinovich et al., 2008). Why one horse may be sensitive to one diet but not other diets may be due to breed, previous management, obesity or other factors; these possibilities should be explored in future studies.

At the beginning of the teff hay study, all horses were fed a mixture of equal parts of all three maturities of hay. This acclimation phase lasted for eight days before the first 12 day experimental period began. Eight days may or may not be an adequate acclimation period for the hindgut microbes to adjust to a new diet. This acclimation period may drastically affect results of future experiments and thus, it is important to assess how much time should be given for adjustment to new feed conditions. In this study, no significant difference was seen in EHSS populations across the three day periods. However, these fecal samples were only collected during the last three days of a 12 day period. If changes occur in the first 9 days, when fecal samples were not collected, significant changes in EHSS populations may have been seen. Our findings suggest that after 10 days on one feed there may be only small fluctuations in hindgut microbe populations in response to diet. Therefore in future studies, longer experimental periods where fecal samples are collected more frequently may reveal a better understanding of the changing microbe populations.

All horses had the highest mean EHSS population at the 1200 hour collection time point. This result may reflect a change in EHSS population growth resulting from a response to the 0700 hour morning feed time. Elevated EHSS population at the 1200 hour time point may also be the result of a circadian effect on nutrient utilization by the horse. While two individual horses had significant differences in EHSS populations (similar to overall findings), the other four horses showed no significant difference at all time points. These results mirror the hay maturity results in showing that the individual horse is the most important factor when evaluating diet. One horse's reaction and EHSS population change may be drastically different than another horse being fed the same diet at the same time.

Miner Institute Study

In this study, a low NSC diet that is routinely prescribed to laminitic prone horses and pasture were compared. Current dogma is that pasture is unsafe to feed laminitic prone horses. However, in this study the pasture diet had the lowest mean EHSS population for all horses and days (Figure 3). Additionally, the mean EHSS population decreased from pasture day 3 to pasture day 14, which had the lowest mean of any dietary treatment or day sampled (Figure 4). These findings reveal that pasture should be re-evaluated as being unsafe for laminitic prone horses through careful use of forage analysis and comparison to available regional hay diets.

Similar to the teff hay study, the change in EHSS populations were the most drastic when comparing individual horses (Figure 5). This reemphasizes that sensitivity of each horse to changes in the hindgut microbes due to diet may be different, even when

fed the same feed under the same conditions. When comparing the results between these two studies, it is interesting to note that the Miner Institute study had an approximately one tenth reduction in %EHSS populations for all horses. The majority of %EHSS values in the teff hay study range from approximately 1-3%, however %EHSS values in the Miner Institute study range from approximately 0.1-0.3%. Several factors may contribute to the difference in the baseline EHSS populations between these two studies. First, each used a different breed of horse, American Quarter Horses in the teff hay study and Morgan horses in the Miner Institute Study. No cross-breed studies have been attempted to date, so it is possible that horses of different breeds may harbor differing populations of EHSS. Management and breeding strategies for the two facilities also differ. Additionally, as horses with frequent symptoms of laminitis are removed from the breeding population at the Miner Institute this potentially changes the genetic susceptibility of the resulting offspring by artificial selection. If EHSS populations are a significant risk factor for laminitis, then strong selection against laminitis may drive lower mean EHSS values in this population.

Conclusions

The population of EHSS is evaluated in these studies as a ratio of EHSS detected to AllBac detected species (%EHSS). Normalizing the EHSS population to total bacterial population, as detected by the AllBac probe, provides an internal control of DNA extraction efficiency and overall bacterial content of the feces. However, we must be cautious in interpreting these results as only changes in EHSS population amounts. Future studies should aim to clarify if increases in %EHSS results from an increase in the

number of EHSS microbes, or whether the AllBac species are decreasing in proportion. Previous studies in oligofructose dosed horses show decreased bacterial cell numbers and a reduction in bacterial species diversity (Milinovich et al., 2007). Additionally, the EHSS probe detects multiple species that should be individually investigated in future studies. However, *S. lutetiensis*, one of the species detected by the EHSS probe, was found previously to be the most prominent microbe prior to onset of laminitis (Milinovich et al., 2006).

In the teff hay study, the late-heading hay with the highest NSC content (compared to the other two maturities), decreased digestibility and decreased voluntary intake was presumed to be unsafe for laminitic prone horses. These findings should be reconsidered as it resulted in the lowest mean EHSS population when compared with the other two maturities of hay. Individual horses react differently to the hay maturities and thus, EHSS population, voluntary intake and digestibility should be carefully evaluated when formulating the safest and most nutritious diet for the horse.

Similarly, all horses included in the Miner Institute study had different reactions and sensitivities to the diets provided. Pasture, especially *ad lib*, is generally considered to be the least safe for laminitic prone horses. However, in this study, the pasture diet resulted in the lowest mean EHSS populations across all days this diet was offered. These findings emphasize that broad generalizations about safety of diet should be reconsidered on an individual horse basis. It is imperative that future studies aim to clarify why the microbial populations within the hindgut of individual horses react differently when presented with various diets in order to predict response to changes in feed and potentially prevent laminitis. These studies have established the sensitivity and

functionality of the assays used, as well as determined a baseline level for healthy horses. The results of this work will provide a good foundation for future research evaluating the microbial populations in laminitic and metabolic syndrome horses.

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