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Original Research

Preliminary Investigation of the Changes in Fecal Streptococcal Population due to Diet and Time of Day in Horses

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ABSTRACT

A growing number of equine studies examine management factors that influence the microbial populations in regions of the gastrointestinal tract using culture-independent molecular techniques. We used quantitative polymerase chain reaction (qPCR) to evaluate changes in fecal streptococcal populations in horses fed different maturities of teff hay with fecal samples collected at 7 AM, 12 PM, and 7 PM. The objective of this study was to use qPCR and species-specific probes for 16S ribosomal DNA to quantify the percentage of equine hindgut streptococcal species (EHSS) relative to the total bacterial load in the feces. Feces from horses fed the most mature teff hay had the lowest %EHSS, and feces collected at 12:00 had the highest %EHSS ($P < .05$). Our interest in investigating %EHSS developed out of research that examined associations between changes in EHSS populations and the onset of laminitis. Although we expected almost no risk of laminitis in these forage fed horses, our hypothesis was that the different carbohydrate fractions in the three maturities of hay would result in differences in %EHSS in horses fed these hay maturities. The supply of carbohydrates, nonstructural or structural, influences the microbial species composition through the gastrointestinal tract. This study highlights the occurrence of measurable changes in %EHSS due to subtle changes in dietary nonstructural carbohydrate and also changes in samples taken at different times of day. This information is useful to others considering investigations of dietary influences on gastrointestinal microbial populations, particularly those that plan to use feces as their main sample medium.

1. Introduction

Equines have evolved over ~55 million years to consume and digest a variety of plant species using a hindgut fermentation strategy [1]. The horse digests plant material with the help of microbial enzymes present throughout the gastrointestinal tract, but most microbial activity is focused in the cecum and colon [2]. This fermentation produces volatile fatty acids which are used by the horse as an energy source [3]. Unfortunately, when horses are fed nonstructural carbohydrate (NSC) rich diets, their relatively small intestinal capacity for digestion of NSC can be overwhelmed. Undigested NSC pass through the small intestine without being absorbed and enter the large intestine, where they are rapidly fermented by adapting hindgut microbial populations [4]. Subclinical acidosis due to this rapid fermentation is thought to negatively impact the selective permeability of the gastrointestinal tract, resulting in colic, colitis, and secondary laminitis [4].

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Although the exact mechanisms remain unclear, systemic inflammation caused by abnormal absorption of toxic factors from the gastrointestinal tract is believed to be important to the etiology of laminitis, an inflammatory condition of the hoof [5]. Grazing on lush pastures and feeding of high NSC diets are perceived to cause >50% of laminitis cases [6]. Potential initiators of laminitis associated with this disruption of gastrointestinal microbial ecology include lamellar ischemia resulting from the absorption of endotoxins released after lysis of gram-negative bacteria [7–10], absorption of vasoactive agents like amines [11–13], or uncontrolled activation of host matrix metalloproteinases used in remodeling and growth of the hoof wall [14,15]. Yet, further research is needed to fully understand the connection between the gastrointestinal tract, systemic inflammation, and destruction of the hoof.

Numerous studies have suggested that changes in gastrointestinal microbial populations immediately precede the onset of experimentally induced laminitis [16,17]. Studying these microbial populations is challenging due to the general inaccessibility of the tract, often requiring collection of samples via nasogastric tube, cannulation, or postmortem dissection [18–20]. Fecal sampling represents a less-invasive approach but may only reflect the population from a portion of the hindgut. Quantification of microbial populations in fecal samples is accomplished using either culture-based or culture-independent molecular approaches. However, culture-based methods are severely limited in that contamination at sample collection is likely, and approximately 70% of equine hindgut microbial species cannot be maintained in culture [21].

Next-generation sequencing technologies can also be used to catalog the diversity of microbial species in the equine hindgut, freshly collected feces, and *in vitro* culture systems [22–26]. Metagenomics studies using sequencing-based technologies provide unprecedented detail in describing a bacterial community [27]. Yet, owing to the relatively high cost and effort required for bioinformatics analyses of sequencing data, the number of samples analyzed in these experiments is often limited, resulting in neglect of potentially important variables like the time of day.

The objective of this study was to apply quantitative polymerase chain reaction (qPCR) detection of 16S ribosomal DNA to quantify the relative bacterial load in horses. We aimed to assess variables of time of day and mild changes in a forage diet (cutting of hay). Given previously reported changes in *Streptococcus* spp. populations in equine feces, we chose to assay the relative abundance of these species using previously published and validated primers and probes [16,25]. Experimental induction of laminitis alters the relative abundance of the bacterial populations detected by this probe, and although they do not provide a complete description of the microbial ecology at play, they are likely relevant measures of overall gut health [16].

2. Materials and Methods

2.1. Animals and Diets

The animal protocols for this study were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (protocol, 29,724). The fecal samples collected for this work were part of a separately published study that examined digestibility and voluntary intake of the forages provided [28]. Housing for the six nonpregnant Quarter Horse mares (12 ± 3 years; 553 ± 39 kg BW; 5.5 ± 0.9 BCS (1–9 scale)) was at the John O. Almquist Research Center in University Park, PA, during January and February, 2009. The mares had access to fresh, clean water and salt blocks (containing only NaCl) for the duration of the experiment. The animals were maintained on rubber mats to facilitate clean sample collection.

The study described here was conducted in conjunction with a separate study investigating the digestibility and dry-matter intake of teff hay [28]. The three maturities of teff hay (*Eragrostis tef*; designated as boot, early-heading, and late-heading maturities) revealed different nutrient compositions and dry-matter digestibilities (Table 1; modified from [28]). The study began with 8 days for the mares to acclimate to experimental conditions and the research center. During this time, the mares received twice daily feedings of a hay mix consisting of equal parts of each maturity in quantities that ensured the mares always had hay available. After the acclimation period, three successive periods lasting 12 days each occurred. During each period, mares were offered one of the three teff maturities and consumed between 8.1 to 9.7 kg/day of dry matter [28]. The study included three dietary treatments (the three teff maturities), three experimental periods, and the six mares used in a replicated balanced Latin square design. During each period, the mares were fed with the experimental hay twice daily (7 AM and 7 PM).

2.2. Sample Collection

Total collection of fecal output from each horse occurred during the three final days of each 12 days experimental period. Feces were removed from the stalls and placed in a closed collection container immediately after defecation. Subsamples were collected into individually labeled 50-mL conical vials three times daily (7 AM, 12 PM, and 7 PM) from the most recently collected fecal output. The timing of the subsamples corresponds to predetermined sampling points for the previously described digestibility trial and was not

Table 1
Dry-matter nutrient composition and digestibility of the boot, early-heading, and late-heading maturities of teff hay fed in this study.

Component	Boot	Early heading	Late heading
DM, %	92.0 ± 0.3	92.1 ± 0.4	92.5 ± 0.6
CP, %	16.4 ± 2.4[a]	10.8 ± 3.4[b]	7.5 ± 0.5[c]
ADF, %	35.7 ± 1.5[a]	40.2 ± 3.4[b]	41.5 ± 1.9[b]
NDF, %	68.1 ± 2.4[a]	71.1 ± 3.7[ab]	70.8 ± 2.0[b]
Lignin, %	3.6 ± 1.9	4.0 ± 0.7	4.0 ± 0.9
Starch, %	0.6 ± 0.4[a]	0.8 ± 0.4[ab]	1.5 ± 0.8[b]
WSC, %	4.8 ± 0.8[a]	6.1 ± 2.0[ab]	6.9 ± 1.2[b]
DMD, %	60.6 ± 2.6[a]	55.3 ± 2.6[b]	51.5 ± 2.6[c]

Abbreviations: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; WSC, water soluble carbohydrate; DMD, dry matter digestibility.
Data are presented as mean ± standard deviation.
[a,b,c] Within a row, means with different superscripts differ ($P < .05$). (Adapted from [28]).