The Utility of Culture Independent Methods to Evaluate the Fecal Microbiome in Overweight Horses Fed Orchard Grass Hay

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Abstract

This dissertation documents efforts to evaluate metabolic variables and the fecal microbiome in adult horses fed grass hay. In the first study, eight Arabian geldings limit-fed an 18% vs. 12% non-structural carbohydrate (NSC) hays in a cross-over design during two 28-day periods were included to evaluate the influence of grass hay NSC on serum insulin and plasma glucose concentrations. Serum insulin concentrations was higher in geldings fed the 18% NSC hay; however, this difference was only detected on day 7 and none of the geldings developed hyperinsulinemia. Blood glucose concentrations did not differ between hay groups.

The second and third studies were extensions of the first and were conducted to use denaturing gradient gel electrophoresis (DGGE) and real-time PCR in evaluating the effect of forage carbohydrates on equine fecal bacteria diversity and abundance, respectively. Fecal microbiomes were similar (80.5-87.9%) between geldings. The abundance of bacteria belonging to the *Firmicutes* phylum increased ($p = 0.02$) in the feces of geldings fed 12% NSC hay (mean 8.06 range [8.03-8.11] $\log_{10}$ copies/g feces) compared to the feces of the same geldings when fed the 18% NSC hay (7.97 [7.97-7.98] $\log_{10}$ copies/g feces). The *Firmicutes* (43.7%), *Verrucomicrobia* (4.1%), *Proteobacteria* (3.8%), and *Bacteroidetes* (3.7%) phyla dominated the fecal microbiomes. This work was the first to report the presence of the *Actinobacteria*, *Cyanobacteria,* and TM7 phyla in the equine fecal or gut microbiome. There was a high abundance (38%) of unclassified bacterial sequences in the gelding fecal microbiome.

In the fourth study, 5 overweight adult mixed-breed mares and 5 adult mixed-breed mares in moderate condition, limit-fed a grass hay, were used to evaluate the effect of body condition

on diet digestibility, plasma and fecal volatile fatty acid (VFA) concentrations, and fecal bacterial abundance. Hay, fecal, and blood samples were taken daily for 4 days after a 10 day adaptation period. A difference in hay digestibility, fecal VFA concentration, or bacterial abundance was not detected between overweight mares and mares in moderate condition. Plasma acetate, a product of microbial fermentation of fiber, was higher in the overweight mare group.

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Attribution

Several colleagues aided in the writing and research behind two chapters presented as part of this dissertation. A brief description of coauthors' contributions are presented here.

Section 3

Section 3 is published in the Journal of Animal Physiology and Animal Nutrition

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Section 5

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List of Abbreviations

ADF	Acid Detergent Fiber
ADFD	Acid Detergent Fiber Digestibility
BCS	Body Condition Score
BMI	Body Mass Index
BW	Body Weight
CF	Crude Fiber
CHO	Carbohydrates
CP	Crude Protein
DE	Digestible Energy
DGGE	Denaturing Gradient Gel Electrophoresis
DGGE-PCR	Generation of PCR amplicons for application in DGGE
DM	Dry Matter
DMB	Dry Matter Basis
DMD	Dry Matter Digestibility
DMI	Dry Matter Intake
DNA	Deoxyribonucleic Acid
ESC	Ethanol-Soluble Carbohydrates
FISH	Fluorescent *In Situ* Hybridization
GC	Guanine + Cytosine
IR	Insulin Resistance
Mcal	1000kcal (1000Calories)
NDF	Neutral Detergent Fiber

NDFD	Neutral Detergent Fiber Digestibility
NSC	Non-Structural Carbohydrates
NSP	Non-Starch Polysaccharides
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
qPCR	Real-Time (Quantitative) PCR
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RTqPCR	Reverse Transcriptase Quantitative PCR
TCA	Tricarboxylic Acid Cycle (Citric Acid Cycle)
UPGMA	Unweighted Pair Group Method With Mathematical Averages
VFA	Volatile Fatty Acids
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine
WSC	Water-Soluble Carbohydrates
ZR	Zymo Research

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Section 1 - Introduction

1.1 - Background

Obesity is a problem in the equine population and is associated with insulin resistance and laminitis. In any given population of horses provided *ad libitum* forage (i.e. horses kept on pasture), some horses become overweight or obese and may subsequently develop laminitis, yet other horses maintain ideal weight and health. The cause of this discrepancy has not been determined.

Hindgut microbes enable the horse to thrive on a forage-based diet; the majority of the horse's daily calories come from microbial fermentation products. In humans, the gut microbiome differs between obese and lean individuals; obese individuals may harbor a more efficient gut microbiome than lean individuals. Due to the reliance of the horse on the hindgut microbes for calorie provision, the presence of distinct differences in the microbiomes of obese and lean horses could have a large impact on how veterinarians manage equine obesity.

Alterations in the equine hindgut microbiome are currently associated with diseases such as laminitis. Carbohydrates similar to those found in forages (i.e. fructans) have been used to induce alterations in the equine hindgut microbiome associated with the onset of laminitis. However, the relationship between forage carbohydrates and the equine hindgut microbiome has not been fully elucidated. Molecular tools can be used to expand identification of equine hindgut microbes beyond traditional culture-based methods and have enabled the scientific community to better understand the equine hindgut microbial population.

The relationship between the overweight condition, forage carbohydrates, and gut microbes has not been evaluated in the horse. A better understanding of the equine hindgut

microbiome is needed to enable researchers and veterinarians understand how this population can be maintained or altered to prevent or treat disease, such as obesity and laminitis.

1.2 - Objectives

The specific objectives of the work presented in this dissertation were to:

- Compare plasma glucose and serum insulin concentrations in adult geldings fed hay with high or moderate non-structural carbohydrates (NSC)
- Evaluate methods for extracting bacterial DNA from equine feces and characterizing its diversity
- Compare fecal bacterial populations in the feces of geldings fed a high and moderate NSC hay
- Characterize the fecal microbiome of Arabian geldings fed grass hay by use of pyrosequencing
- Compare grass hay digestibility, products of microbial fermentation, and abundance of fecal *Firmicutes*, *Bacteroidetes*, and the fibrolytic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* in overweight mares vs. mares in moderate condition

1.3 - Hypotheses

My hypotheses are:

- Arabian geldings would have higher plasma glucose and serum insulin concentrations when fed high (18%) vs. moderate (12%) NSC Orchard Grass hay
- Arabian geldings would have a unique fecal bacterial community fingerprint and higher abundance of fecal *Firmicutes* members when fed a high (18%) vs. a moderate (12%) NSC hay

- Characterization of the fecal microbiome of Arabian geldings fed variable carbohydrate hay, by use of pyrosequencing, would expand on the current knowledge of equine hindgut and fecal bacteria
- Overweight mares would have higher hay digestibility, higher fecal and plasma acetate concentrations, lower abundance of *Bacteroidetes*, and higher abundance of *Firmicutes* and fibrolytic bacteria than mares in moderate condition

1.4 - Studies

In the first study, 8 overweight Arabian geldings were used to evaluate the effect of feeding a high (18%) or moderate (12%) non-structural carbohydrate (NSC) hay on plasma glucose and serum insulin concentrations. Horses were individually housed and limit-fed each of the two hays, split between two daily feedings, for 28 days in a cross-over design. Blood samples were collected prior to the morning meal every 7 days for determination of plasma glucose and serum insulin.

The second and third studies were extensions of the first study. In the second study, fecal samples were collected from the Arabian geldings during the last week of the first feeding period and throughout the second feeding period. Fecal bacteria communities of these geldings were compared using 16S rDNA fingerprinting by denaturing gradient gel electrophoresis (DGGE) and real-time PCR (qPCR) targeting specific bacterial phyla. For the third study, two fecal samples from an obese and non-obese Arabian gelding, each representing the last week of each feeding period when the horses were fed the 18% and 12% NSC hays, were evaluated by use of 16S rDNA amplicon pyrosequencing to identify bacterial membership of the fecal community.

The fourth study, 10 adult mixed-breed mares were selected from the VMRCVM teaching herd. Horses were included into the overweight (n=5) or moderate condition (n=5) group based on their phenotype over the last 3+ years. Horses were housed individually and

limit-fed Orchard Grass (*Dactylis glomerata*) hay at 2% body weight (as fed) daily for 14 days. The first 10 days served as an acclimation period. During the last 4 days of the study hay, blood, and fecal samples were collected for determination of hay digestibility, plasma and fecal volatile fatty acid (VFA) concentrations, and abundance of total bacteria, *Firmicutes*, *Bacteroidetes*, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*.

Section 2 - Review of the Literature

2.1 - History of the Horse

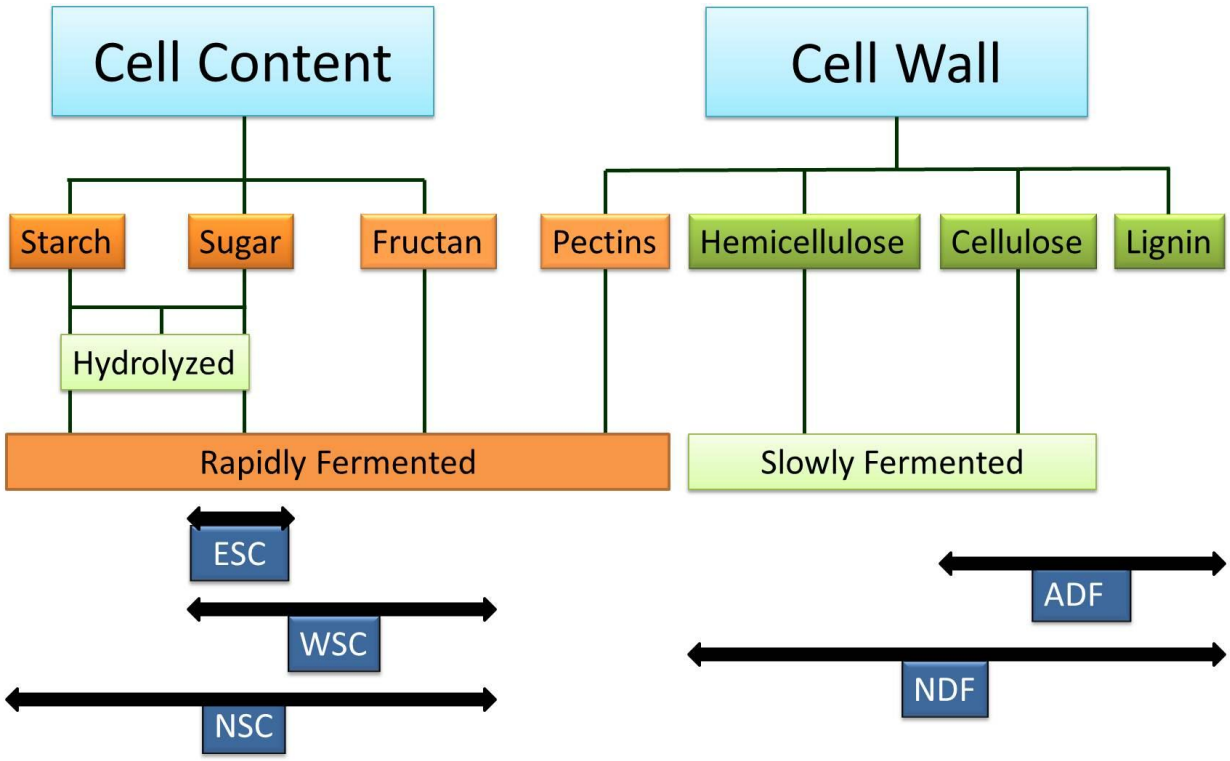
The horse evolved consuming forage approximately 1 million years ago in conjunction with the emergence of grasslands (Al Jassim and Andrews, 2009). The horse was domesticated somewhere around 5000BC mostly for meat, but with time, the horse was used more for work and transport (Kavar and Dovc, 2008). Horses may have evolved in grassland habitats because they were seeking food that suited their gastrointestinal physiology. Alternately, the evolution with grasslands may have shaped the development of this gastrointestinal physiology. Regardless, the horse of today still thrives on a forage-based diet due to fermentative activities of microorganisms residing in the gastrointestinal tract, particularly the large intestine.

2.2 - The Equine Diet

Forages make up 50-100% of a horse's diet; up to 50% of a horse's diet may include concentrates (i.e. grains and commercial feeds) if the forage does not meet energy and protein requirements alone. Common forages fed to horses include either cool-season grasses (e.g. Tall Fescue [*Festuca arundinacea*], Orchard Grass [*Dactylis glomerata*] and Timothy [*Phleum pratense*]) or warm-season grasses (e.g. Bermuda grass [*Cynodon dactylon*]) and cool-season legumes (e.g. Alfalfa [*Medicago sativa*], clovers [e.g. *Trifolium pratense*]); although this may vary with geographic location. Tall Fescue (*Festuca arundinacea*) is the dominant forage found in grasslands (and thus horse pastures) across the United States (Ball et al., 2007). Forages contain both digestible carbohydrates (i.e. sugar and starch) and indigestible carbohydrates, designated as fibers (i.e. cellulose, hemicellulose, pectin and fructan) (Figure 1). Forage carbohydrates may be described by a variety of methods: structure (mono/disaccharide, oligosaccharide, and polysaccharide), function in the plant (structural vs. non-structural),

digestibility (digestible vs. non-digestible), and fermentability (slow vs. rapid) (Hoffman et al., 2001; James et al., 2003; Niness, 1999; NRC, 2007) (Figure 1).

Figure 1 – *Forage Carbohydrates*



ADF: acid detergent fiber; Hydrolyzed carbohydrates - those digested by mammalian enzymes and absorbed in the small intestine; ESC: ethanol soluble carbohydrates; NDF: neutral detergent fiber; NSC: non-structural carbohydrates; WSC: water-soluble carbohydrates

Cellulose and hemicellulose are the most abundant carbohydrates in the equine forage diet, representing 33% (calculated as ADF – lignin) and 24% (calculated as NDF – ADF), respectively, of grass hay dry matter, while mono/disaccharides and starch collectively represent 11-25% of grass hay dry matter (calculated from mean values from a commercial forage lab

(Equi-Analytical, 2004a)). Fibers (e.g. cellulose and hemicellulose) are complexes of monosaccharides linked by β-glycosidic bonds, which can not be degraded by mamallian enzymes in the small intestine. Therefore, fibers travel to the cecum and colon (hindgut) where they are fermented by microbes, particularly bacteria (Bergman, 1990). Slowly fermented carbohydrates (e.g. cellulose) promote a near neutral hindgut luminal pH (~6) (Argenzio et al., 1974).

Other carbohydrates present in cool-season grasses include those hydrolyzed by the host in the small intestine (i.e. sugars and starch) and soluble fibers such as fructans (James et al., 2003; Niness, 1999). The term non-structural carbohydrate (NSC) represents the sum of sugars, starches, and fructans in cool-season grasses. Sugar and starch are digested in the small intestine by mammalian enzymes and positively influence postprandial serum insulin concentrations in the horse (Jose-Cunilleras et al., 2004). Although molasses and grains are concentrated sources of sugars and starches, respectively, forages also contain sugars and starches. Sugars (mono/disaccharides) are the products of photosynthesis and the concentration of sugars in forage can vary within and between day depending on environmental conditions (i.e. rain, sun, temperature).

Warm season grasses (e.g. Bermuda grass [*Cynodon dactylon*]) and legumes (e.g. Alfalfa [*Medicago sativa*]) store energy in the form of starch in chloroplasts and do not produce fructans; starch production and storage is limited when chloroplasts become saturated (Longland and Byrd, 2006). Cool-season grasses (e.g. Orchard Grass [*Dactylis glomerata*]), store energy in the form of fructans in the stems and leaves and this process is not self-limiting (Longland and Byrd, 2006). Therefore, cool-season grasses generally have a higher total NSC concentration

than warm-season grasses due to the ability to accumulate high concentrations of fructans (Pollock and Cairns, 1991).

2.3 - Equine Hindgut Physiology

2.3.1 - Structure and Function

Horses are monogastric animals; therefore, ingesta passes through the esophagus into a simple stomach and small intestine. Proteins, fats, disaccharides, and starch polysaccharides are digested by enzymes present in the stomach and small intestine (i.e. protease, lipase, disaccharidases, and amylase, respectively) and absorbed in the small intestine as occurs in other monogastrics (i.e. human and dog). However, due to the high fiber nature of the horse's diet, the majority of ingested substrate passes on to the hindgut. The horse's large intestine is made up of the cecum, large colon, and small colon, with the small colon representing the transverse and descending colon, the site for final water absorption and fecal ball formation (Merritt, 2003). Unique to hindgut fermenters (i.e. horse, rabbit and rat) are the large and voluminous cecum and large colon (Stevens and Hume, 1998). The equine large colon, a dilation and extension of the human equivalent of the ascending colon, is subdivided into, in aborad order, right ventral colon, left ventral colon, left dorsal colon, and right dorsal colon (Van Weyenberg et al., 2006). The equine hindgut (cecum and large colon) comprises the majority (66-70%) of the equine gastrointestinal tract volume (Argenzio, 1975; Reed et al., 2004) and fibrous plant material spends 18-38 hours in the hindgut; representing 75-85% of mean gastrointestinal retention time (Ross et al., 1986; Van Weyenberg et al., 2006). The equine hindgut is critical for the ability of this herbivore to thrive on a fibrous plant diet (Hintz et al., 1978).

2.3.2 - Hindgut Microbes and Fermentation

The horse relies on gut microbes to thrive on a high fiber diet and alterations in the equine gut microbiome may have negative consequences to the health of the horse. Although the primary site of microbial fermentation in the horse is the hindgut (Glinsky et al., 1976), some microbial fermentation occurs in the equine small intestine (Argenzio et al., 1974; Mackie and Wilkins, 1988; Moore-Colyer et al., 2002). The hindgut is an ideal anaerobic fermentative chamber for a complex anaerobic microbial population due to the availability of substrate for fermentation (i.e. fiber), regulation of temperature, elimination of waste products, and low oxygen concentration. Equine hindgut bacteria, especially those with fibrolytic metabolism, hydrolyze the ß-glycosidic bonds of fibers thereby releasing the monosaccharide subunits. Monosaccharides are then fermented by other hindgut bacteria into molecules; such as volatile fatty acids (VFA), which are used by the horse. Gaseous byproducts of microbial fermentation include carbon dioxide, hydrogen, and methane (Titus and Ahearn, 1992; Williams et al., 2001).

2.3.3 - Products of Microbial Fermentation and Their Absorption

The dominant products of microbial fermentation are the volatile fatty acids acetate, propionate, and butyrate, which represent 60-80%, 10-20%, and $\leq$10% of total VFAs, respectively, in the equine hindgut (Argenzio et al., 1974; Elsden et al., 1946; Glinsky et al., 1976; Hintz et al., 1971; Hussein et al., 2004).

Volatile fatty acids can be found in two forms:

1) Anion – aka. Ionized/polar form
    a. Acetate (CH_3COO-)
    b. Propionate ($CH_3\ CH_2COO$-)
    c. Butyrate ($CH_3\ CH_2\ CH_2COO$-)

2) Acid – aka. Ester/unionized/protonated form

   a. Acetic acid (CH_3COOH); p*K*a = 4.79

   b. Propionic acid ($CH_3\ CH_2COOH$); p*K*a = 4.87

   c. Butyric acid ($CH_3\ CH_2\ CH_2COOH$); p*K*a = 4.82

There are two general mechanisms by which VFAs are absorbed across the cecum and colon enterocytes (Argenzio et al., 1977; Argenzio et al., 1974; Titus and Ahearn, 1992):

1) Passive diffusion of the acid form of VFAs across the enterocyte luminal boarder.

2) Active carrier-mediated transport of anion-form into the enterocyte.

Volatile fatty acids are weak acids (pK $\leq$4.9) thus are primarily in their anion form (i.e. acetate; CH_3COO-) in the nearly neutral normal hindgut lumen pH (6-6.7) (Argenzio et al., 1974; Bergman, 1990; Merritt, 2003; von Engelhardt et al., 1998). To convert the anion into acid for absorption, carbon dioxide, a byproduct of microbial fermentation, is hydrated to H_2CO_3, which subsequently donates a hydrogen (H^+) atom to the VFA anion (i.e. CH_3COO-) to create the acid form (i.e. CH_3COOH) that can passively cross the enterocyte luminal (apical) border. Volatile fatty acid absorption is negatively correlated with pH (Bergman, 1990); as pH drops, VFA absorption increases as the anions become protonated (addition of H^+). Furthermore, VFA absorption is positively associated with the concentration of a given VFA in the lumen (i.e. acetate concentrations increase acetate absorption) (Bergman, 1990).

Absorption of the anion form via the enterocyte luminal border occurs by ways of sodium/hydrogen transport and in exchange for bicarbonate at the enterocyte luminal border (Argenzio, 1975; Argenzio et al., 1977; Topping and Clifton, 2001). Carrier mediated VFA transport mechanisms may be more important when the gut lumen is more alkaline (pH~7) (Dijkstra, 1994) when less hydrogen protons are available.

Argenzio, Southworth, and Stevens (1974) reported that VFA absorption per unit of area increased along the gastrointestinal tract: cecum (4.1 $\pm$0.7 mmol/cm^2), ventral colon (4.8 $\pm$0.6 mmol/cm^2), dorsal colon (5.6 $\pm$0.9 mmol/cm^2), and small colon mucosa (7.8 $\pm$0.9 mmol/cm^2). Once in the blood, acetate may be aerobically oxidized and directly used for energy (Pethick et al., 1993) or stored as triglyceride in adipose and skeletal tissue (Suagee et al., 2010). Most absorbed propionate is converted into glucose by the liver and provides 50-61% of blood glucose in horses (Simmons and Ford, 1991). Butyrate is the preferred energy substrate for colonocytes and thus plays an important role in maintenance of hindgut health. Therefore, plasma VFA concentrations in the jugular vein will not accurately represent microbial VFA production or even portal vein VFA concentrations, particularly for propionate and butyrate.

Vermorel and Martin-Rosset (1997) calculated 80% of absorbed energy in forage-fed horses to be derived from VFAs. Microbial fermentation in the cecum and colon contribute to roughly 1/3 and 2/3 of total daily calories, respectively (Glinsky et al., 1976). Pethick *et al*.(1993) evaluated fractional extraction of nutrients in the hind limb of adult thoroughbred horses and reported that acetate oxidation represented 32% of total nutrient metabolism in the hind limb of the forage-fed horses. Therefore, hindgut microbial fermentation provides a significant portion of daily energy requirements.

2.4 - Equine Obesity

Obesity is prevalent in the equine population; in a prospective study of 300 adult horses in Southwest Virginia, 32% of adult horses were overweight and 19% were obese (Thatcher et al., 2008). Co-morbidities of obesity in horses include reduced reproductive performance (Sessions et al., 2004; Vick et al., 2006), reduced evaporative cooling and reduced athletic performance (Sillence et al., 2006), and increased risk for laminitis (Carter et al., 2009b; Treiber

et al., 2006). The simplistic cause of obesity in horses is consumption of too many calories, expenditure of too few calories relative to metabolic need, or both. Perhaps the obese horse eats more or exercises less; or perhaps the obese horse has a more efficient hindgut microbiome and thus greater diet digestibility than the lean horse.

2.4.1 - Diagnosis of Equine Obesity

Obesity is defined by the accumulation of an abnormally large quantity of adipose tissue. Obesity in horses is most commonly diagnosed using a subjective nine-point body condition scoring system with a score of 1 indicating emaciation and a score of 9 indicating obesity (Henneke et al., 1983). Henneke *et al*.(1983) and Vick *et al*.(2007) reported that BCS positively correlated (R^2 = 0.65 and 0.90, respectively) with percent body fat in horses.

Objective methods for assessing obesity have also been described (Carter et al., 2009a; Donaldson et al., 2004; Westervelt et al., 1976). For example, Westervelt *et al.* (1976) reported that rump fat thickness of, determined by use of ultrasound, strongly correlated with actual rump fat thickness (R^2 = 0.85) and percent body fat (R^2 = 0.93) in horses. Furthermore, a rump fat thickness of 1.55cm represented 15.9% carcass fat mass in the horses. Body mass index (BMI) calculations have been described for horses (Donaldson et al., 2004); however, BMI does not appear to strongly correlate with fat mass or BCS (Donaldson et al., 2004; Henneke et al., 1983).

2.4.2 - Calorie Consumption – Variation in Dry Matter Intake

Crozier *et al*.(1997) evaluated voluntary intake of alfalfa, tall fescue and Caucasian bluestem hays in Arabian geldings and reported that voluntary DMI was negatively associated with forage NDF concentration. However, alfalfa hay had lower NDF (%DM) than the Tall fescue or Caucasian bluestem hays and thus the influence of forage type on palatability could have influenced intake and obscured the effect of NDF on intake. Staniar *et al.*(2010) evaluated voluntary intake of Teff hay at various maturity stages and reported that Quarter Horse mares

consumed more (1.8% BW/day) of the immature Teff hay (16.5% CP, 35.7% ADF and 68.1% NDF) than the mature Teff hay (7.5% CP, 41.5% ADF and 70.8% NDF) (1.5% BW/day). When fed *ad libitum*, this difference in voluntary intake amounts to an intake difference of 1.7kg/d and a digestible energy difference of 3.2Mcal/d for a 553kg mare. Additionally, digestible energy content of grass hay and grass pasture can vary by 20% (Equi-Analytical, 2004a) and 30% (Equi-Analytical, 2004b), respectively. Therefore, horses at maintenance can easily consume too many calories when fed a moderate to good quality forage and thus store the excess energy in adipose tissue.

2.4.3 - Adipobiology

Adipose tissue was traditionally considered solely a source of stored energy. Recently, adipose has been classified as an endocrine organ that regulates metabolism through release of adipokines; such as leptin and adiponectin. Leptin is positively correlated to fat mass and regulates feed intake in mice (Campfield et al., 1995). Knockout *ob/ob* mice, used as a model for obesity research, do not synthesize leptin, consume more food, and become fatter than wild type mice (Ingalls et al., 1950). Leptin down regulates food intake by stimulating synthesis of and sensitivity to satiety factors (i.e. anorexigenic hormones [e.g. cholecystokinin and melanocortin]) and inhibiting orexigenic hormones (e.g. neuropeptide Y) (Donaldson et al., 2004; Schwartz et al., 2000; Woods et al., 2000). Despite leptin's role in food intake reduction, obese individuals may maintain intake possibly due to leptin resistance. Buff *et al*.(2002) and Carter *et al*.(2009a) reported that serum leptin concentrations were positively correlated with body condition score and adiposity in horses. The upregulation of satiety/anorexogenic hormones in the obese state suggests a loss of function either due to altered hormone function or decreased sensitivity at the target tissue level.

Adipose tissue also produces inflammatory cytokines and obesity is considered as a state of chronic inflammation (Hotamisligil, 2006; Vick et al., 2007). Inflammation has been linked as a causative factor behind obesity-associated insulin resistance in the horse. Vick *et al*.(2007) reported that serum interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) in adult mares were positively correlated with BCS and % body fat in adult horses. Furthermore, the increased circulating leptin associated with obesity may positively influence the chronic inflammatory state associated with obesity (Bleau et al., 2005). Leptin is positivly associated with IL-1beta and TNF-alpha gene expression and leptin deficiency in mice is associated with reduced clinical signs of arthritis (Busso et al., 2002), an example of an inflammatory condition that is associated with obesity.

2.5 - Equine Obesity Comorbidities

2.5.1 - Equine Insulin Resistance

Increased adiposity in horses is positively associated with the development of insulin resistance in horses (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007). Reduced insulin sensitivity is associated with hyperinsulinemia (>40μIU/L, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY) in non-fasted forage-fed horses. In a Southwest Virginia cohort of horses, 10% of total horses were hyperinsulinemic and the majority (60%) of hyperinsulinemic horses were obese (BCS 7.5/9) (Geor et al., 2007). Pro-inflammatory adipokines (Shoelson et al., 2006) negatively impact insulin signaling. Furthermore, accumulation of intracellular fatty acids can interfere with cellular signaling and thus insulin sensitivity. Accumulation of fatty acids or triglyceride in the myocyte cytosol activates protein kinase C which inhibits the insulin receptor as well as translocation of the Glut-4 receptor subsequently causing skeletal muscle insulin resistance (Kelley and Mandarino,

2000). Frank *et al.*(2006) reported that serum leptin positivily correlated with insulin resistance in obese horses and thus can be used as a screening method, in addition to resting serum insulin concentrations and BCS, for insulin resistance. Interestingly, insulin resistance in the horse does not appear to progress to Diabetes Mellitus (Johnson, 2002; Johnson et al., 2005) in contrast to other species.

2.5.2 - Equine Laminitis

Laminitis is a painful and debilitating condition of the equine hoof characterized by a breakdown of the bond between the connective tissue of the coffin bone (distal phalanx) and inner hoof wall. Once the bond is compromised, the coffin bone rotates and sinks within the hoof capsule, which causes the horse to become severely lame and recumbent in severe cases. A body condition score of 7/9 – 9/9 is a risk factor for laminitis in ponies fed forage (Carter et al., 2009b; Treiber et al., 2005b). Obesity or increased adiposity is thought to increase a horse's risk of laminitis by multiple mechanisms including alterations in laminar vascular tone, promotion of a pro-inflammatory state, and elevated serum insulin concentrations (Frank, 2009; Frank et al., 2010). Carter *et al.*(2009b) reported that elevated plasma insulin (> 32 μU/L) was a predictor of laminitis in pasture-fed ponies. Treiber *et al.*(2006) reported that blood insulin concentrations of pastured ponies were higher in currently laminitic (103.7 μU/L) and previously laminitic (21.5 μU/L) ponies than ponies without laminitis episodes (12μU/L).

Other conditions, such as gastrointestinal disease, systemic infection, and consumption of large volumes of non-structural carbohydrates (NSC), contribute to the onset of laminitis (Bailey et al., 2002; Garner et al., 1975; van Eps and Pollitt, 2006). Laminitis may be induced experimentally by oral administration of starch (Garner et al., 1975), oligofructose (fructan) (van Eps and Pollitt, 2006), Black Walnut tree (*Juglans nigra*) extract (Minnick et al., 1987), and

supraphysiologic parenteral insulin infusion (Asplin et al., 2007). The majority of naturally occurring laminitis cases are associated with *ad libitum* pasture consumption (Hinckley and Henderson, 1996; USDA, 2000). Laminitis prevention is critical as 5% of cases die or are euthanized (USDA, 2000). Obese horses may be at greater risk of laminitis associated with excessive NSC consumption. Obesity prevention/treatment and dietary intervention to limit NSC consumption should reduce the incidence of obesity and co-morbidities such as laminitis.

2.6 – The Role of Forage Carbohydrates in Insulin Resistance and Laminitis

2.6.1 - Forage and Insulin Resistance

Chronic consumption of highly digestible carbohydrates, in the form of grain-based starch and sugar, is positively associated with insulin resistance in horses (Treiber et al., 2005a). A common dietary suggestion for the obese, insulin resistant, and/or laminitic horse is to restrict digestible carbohydrates by removing grain from the diet and feeding a low (~10%) NSC hay to both reduce the insulin response to a meal and reduce calories in the diet (Frank et al., 2010). The glycemic response to a meal does not appear to fully explain the relationship between soluble carbohydrates in the equine diet and reduced insulin sensitivity and the role of forage fructans on equine health is less clear. Fructan is a fiber and thus should be minimally or not hydrolyzed in the small intestine; furthermore, fructose results in a smaller insulin response to a meal. Baily *et al.*(2007) reported that oral administration of levan-type fructan to non-obese (BCS 5/9) ponies with a history of laminitis resulted in reduced insulin sensitivity. Furthermore, serum insulin concentrations were higher in the same cohort of ponies when allowed *ad libitum* access to fresh pasture (138g fructan/kg DM) as compared to dry hay (34g fructan/kg DM).

2.6.2 - Forage and Laminitis

If large amounts of grain (>5 pounds/feeding or >0.4% BW in starch/feeding) are fed to horses, the sugars and starches can escape the small intestine and become rapidly fermented by

microbes in the hindgut into lactic acid (Glinsky et al., 1976; Potter, 1992). The accumulation of lactic acid and reduction of pH in the equine hindgut disturbs the healthy hindgut microbial population and may lead to severe illness in the horse, such as laminitis (Garner et al., 1975; Mungall et al., 2001).

Inulin-type fructans (Ritsema and Smeekens, 2003) from chicory root (*Cichorium intybus*) are rapidly fermented in the equine hindgut and can be used to induce laminitis (Milinovich et al., 2008). The dose of inulin-type fructan (7.5g/kg BW) used to induce laminitis was first described by van Eps and Pollitt (2006). However, cool-season grasses produce mostly phlein or levan-type fructans (Pollitt 2003). Levan-type fructans (Ritsema and Smeekens, 2003) in forages are postulated to be the cause of pasture-associated laminitis (Pollitt et al., 2003; Treiber et al., 2006; USDA, 2000) in horses because cool-season grasses accumulate fructans and intake of pasture forage is not typically restricted. Levan-type fructans are longer molecules (10-260 glucose and fructose polymers) with beta 2,6-linked bonds as compared to the shorter inulin-type fructan molecules ($\leq$10 glucose and fructose polymers) with beta 2,1 bonds (Longland and Byrd, 2006; Pollock and Cairns, 1991). Based on an estimated intake of 2% BW per day in total dry matter and fructan concentration of up to 28-50% forage DM (Pollitt et al., 2003) in the stems and leaves in cool-season grasses (Longland and Byrd, 2006), a 500kg horse in the United Kingdom could consume up to 2.8-5.0kg fructan in a 24-hr period, which is less than the single fructan dose used to induce laminitis in horses. A 500kg horses would have to consume 3%BW per day in total dry matter of a forage with 50% DM fructan to match the laminitis induction dose and this dose would be consumed throughout the day and not in a single bolus. Kalck *et al*.(2009) induced laminitis with a lower dose of fructan (5g/kg BW). However, grasses in the US contain an average 3% fructan (calculated as WSC – ESC of grass hay dry

matter from mean values from a commercial forage lab (Equi-Analytical, 2004b)), which provides about 0.3kg fructan per day to a 500kg horses and this is lower than the priming dose (1g fructan/kg BW) for laminitis induction studies (Kalck et al., 2009; Milinovich et al., 2006; van Eps and Pollitt, 2006). There are no studies to date evaluating the role of levan-type fructans and laminitis and there is little published work evaluating the relationship between forage carbohydrates collectively and laminitis (Bailey et al., 2007).

2.7 - The Gut Microbiome

The gastrointestinal tract (gut) of vertebrates and invertebrates is inhabited by microorganisms (gut microbes) which are predominantly bacteria. In humans and rodents, the number of microbial cells is 10-fold higher than cells of the host (Bäckhed et al., 2005; Savage, 1977) and the number of genes in the gut microbiome is estimated to exceed the number of host genes by a factor of 100 (Xu and Gordon, 2003). The genomes of the microbes within the gut are collectively called the gut microbiome (Turnbaugh et al., 2007) and are dominated by the *Firmicutes* phylum in multiple species including humans (Larsen et al., 2010), pigs (Guo et al., 2008a), and ruminants (Shanks et al., 2011). Essential roles of these microbes in the mammalian host include digestion of fibers, synthesis of vitamins, biotransformation of conjugated bile acids, dietary oxalate degradation, stimulation of the immune system, inhibition of pathogenic bacteria, alteration of host fatty acid composition, and maintenance of colonocyte health (Bergman, 1990; Chen et al., 1999; Duncan et al., 2002b; Gibson and Roberfroid, 1995; Hylemon and Harder, 1999).

The bacterial ecosystem within the gut is established at birth and is influenced by bacteria residing in the dam's rectum, vagina, cervix, mammary gland, and milk as well as other environmental sources (Mackie et al., 1999). However, the *primary* hindgut bacteria in adults

are not those found in the environment (Ley et al., 2006b). The gut microbiome develops from birth to ~3y/o in humans after which the microbiome becomes more stable into adulthood. In mice and humans, mother/offspring and siblings have more simillar gut and fecal microbiomes than unrelated individuals, reflecting a long-term impact of initial colonization (Turnbaugh et al., 2009). This complex population is important for the maintenance of host health as disturbances or shifts in the bacterial population have been associated with disease in the host.

2.7.1 - Impact of Diet on the Gut Microbiome

Diet influences the gut microbiome in multiple species (De Filippo et al., 2010; Hildebrandt et al., 2009; Middelbos et al., 2010; Tajima et al., 2001). The abundance of *Firmicutes* members increased in the feces of dogs fed a commercial diet with beet pulp as compared to a control diet with brewers rice (Middelbos et al., 2010). In the rumen of cows, a switch from a forage diet to a high grain diet (to a lower fiber diet) was associated with reduced abundance of fibrolytic bacteria and *Treponema bryantii* along with an increased abundance of *Prevotella* spp. and *Streptococcus bovis* (Tajima et al., 2001). Children in rural Africa consuming high fiber diets had a higher abundance of *Bacteroidetes* phyla members and a lower abundance of *Firmicutes* phyla members as compared to European children (De Filippo et al., 2010). While the diets are very different between these two cohorts, the influence of environment can not be ruled out. In rats, feeding a high fat vs. standard commercial diet caused an increase in *Firmicutes* and *Proteobacteria* abundance along with a reduction in *Bacteroidetes* abundance (Hildebrandt et al., 2009). The effect of feeding a high fat diet to rodents may also represent the effect of feeding a low carbohydrate as carbohydrates are likely the macromolecule typically replaced by fat in these diets.

2.7.2 - Obesity Microbiome

Over the last decade, researchers have identified human microbial communities associated with the skin, airway, urogenital tract, oral cavity, and gut (Peterson et al., 2009). A link between obesity and hindgut microbes has been established and extensively studied. Distinct shifts in hindgut microbial populations have been described in obese humans (Ley et al., 2006a), rodents (Ley et al., 2005), pigs (Guo et al., 2008b), and dogs (Handl, 2010). Obesity-associated gut microbial shifts in humans and rodents have been characterized by an increased abundance in the *Firmicutes* phyla members and decreased abundance of *Bacteroidetes* phyla members (Ley et al., 2005; Ley et al., 2006a; Turnbaugh et al., 2009; Turnbaugh et al., 2006). In pigs, the abundance of *Bacteroidetes* phyla members is negatively correlated with back fat thickness (Guo et al., 2008a). Furthermore, the gut microbiome of obese humans is less diverse than in lean counterparts (Turnbaugh et al., 2009) and the abundance of *Bacteroidetes* is positively associated with weight loss when humans are placed either on a fat or carbohydrate-restricted diet (Ley et al., 2006a). However, the *Firmicutes: Bacteroidetes* ratio in obese vs. non-obese humans differs among studies; these differences may be due to the use of different methodologies. Turnbaugh *et al*. (2009) observed a positive correlation with the *Firmicutes: Bacteroidetes* ratio and obesity when bacteria were identified with pyrosequencing. However Schwiertz *et al*. (2010) and Duncan *et al*. (2008) did not find the same correlation when bacteria were identified with fluorescent in situ hybridization. The causal relationship between gut microbes and the obese host has not been fully elucidated as there is an influence of host on gut microbiome (Ley et al., 2006b) in addition to the influence of gut microbiome on host (Kalliomaki et al., 2008). Therefore, whether changes in the gut microbiome result in or are a marker of obesity has yet to be fully determined.

2.7.3 - Causative Relationship Between Gut Microbes and Obesity

Gut microbes may promote the obese state through enhancing energy extraction from the host's diet (Samuel and Gordon, 2006) or augmenting host fat storage (Bäckhed et al., 2004; Samuel and Gordon, 2006). Microbial production of volatile fatty acids provides about 7-10% of the energy requirements of humans and rodents (Cummings, 1981; McNeil, 1984; Titus and Ahearn, 1992; von Engelhardt et al., 1998; Xu and Gordon, 2003). However, this contribution varies depending on the specific bacterial community members in the gut (Ley et al., 2005). Gnotobiotic (germ free/no gut microbes) rats retain less dietary energy than conventionally raised rats (Wostmann et al., 1983). Obesity in rodents is associated with reduced fecal energy which infers more energy extraction by the host perhaps due to gut microbes (Turnbaugh et al., 2006). G protein-coupled receptors (Gpr) are expressed throughout the large intestine; VFAs bind to these receptors and stimulate leptin expression. Although chow intake of *Gpr* gene deficient mice do not differ from control mice, fecal energy is higher in *Gpr* gene deficient mice (Samuel et al., 2008).

Conventionally raised mice have 42% greater body fat than gnotobiotic mice; and gnotobiotic mice develop obesity and insulin resistance within 14 days of populating their gut with the microbiome from conventionally raised mice (Bäckhed et al., 2004; Bäckhed et al., 2007). The presence of two specific microbes, *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii*, a methanogenic archaea, are associated with increased body fat and plasma acetate in the mouse (Samuel and Gordon, 2006; Samuel et al., 2008). Methanogenic archaea utilize hydrogen; therefore, the presence of methanogenic archaea may enhance fibrolytic function by scavenging this waste product of fiber fermentation. Furthermore, colonization of the murine gut with *Bacteroides thetaiotaomicron* and the archaea *Methanobrevibacter smithii* is associated with increased fat storage through secretion of fasting-

induced adipocyte factor (lipoprotein lipase inhibitor) in the gut epithelium and increased monosaccharide uptake by the gut with subsequent increased *de novo* hepatic lipogenesis enzymes (Bäckhed et al., 2004; Bäckhed et al., 2007; Samuel et al., 2008).

2.8 - Culture Independent Evaluation of the Gut Microbiome

2.8.1 - Culture Independent vs. Culture Independent Microbial Evaluation

Only 30% equine hindgut bacteria can be identified by traditional culture techniques (Mackie and Wilkins, 1988), which is similar to reports in humans (Mackie and Wilkins, 1988; Suau et al., 1999). The challenges in culturing gut bacteria include: 1) presence of obligate anaerobes in the gut, 2) interactions between gut bacteria, and 3) interactions between gut bacteria and the host (i.e. host's diet and immune system) (Sartor et al., 2007). Furthermore, cultivatable bacteria will not behave the same in culture as in their complex community (Rediers et al., 2005) and thus may alter their function in culture (Marco et al., 2009). Culture-independent methods are therefore needed to evaluate complex microbial ecosystems.

There is no single gold standard method for evaluating a complex microbial population and thus multiple methodologies are often employed. Culture-independent technologies (Juste et al., 2008); such as, high-throughput sequencing (e.g. pyrosequencing (Gill et al., 2006; Turnbaugh et al., 2008)), clone-library generation (Daly et al., 2001) and quantitative PCR (Guo et al., 2008a)) have been utilized to evaluate microbial communities in a variety of environments. The advent of molecular tools provides a much broader description the bacterial community and eliminates the bias of culture-dependent techniques.

2.8.2 - Sample Collection (Step 1)

The first challenge in evaluation of the gut microbiome of any species is to select appropriate samples. Hindgut samples can be collected in horses via a large intestine cannula

(Lopes et al., 2004; Medina et al., 2002; Muhonen et al., 2009) and surgical collection; however, both methods are invasive and impose surgical risk on the horse. Postmortem sample collection (Bailey et al., 2002; Daly et al., 2001) is an alternative; however, this method is limited to a one-time collection.

Fecal samples are low-cost and non-invasive method for sampling gut microbes. Furthermore, due to the ease of sampling, serial evaluation of fecal microbial communities over time may be performed without compromising the health of the host. In humans, the similarity between the fecal and gut microbiome has been evaluated by both culture and culture-independent methods. Moore *et al.*(Moore et al., 1978) described similar cultivated bacteria in the colon and fecal samples in humans collected immediately postmortem. Eckburg *et al*. (2005) evaluated the large intestinal mucosal and fecal microbiome of three human subjects. The authors reported that within subject mucosal microbiomes along the cecum and large intestine are similar, but that the fecal microbiome differed from the mucosal samples. The difference between fecal and mucosal communities reported by Eckburg *et al*. (2005) may be falsely increased due to the delayed time (1 month) which the fecal samples were obtained after the colonoscopic mucosal biopsies. Daly *et al*. (2001; 2011) reported that bacterial populations are relatively consistent throughout various regions of the equine hindgut. Furthermore, the abundance of fibrolytic bacteria did not differ between the cecum, colon and feces of horses (Hastie et al., 2008), which provides some evidence of the utility of feces as an appropriate non-invasive means of sampling the equine hindgut microbiome

Milinovich *et al*. (2007) used Gram staining and fluorescence *in situ* hybridization (FISH) to evaluate both the effect of inulin-type fructan administration on equine cecal and fecal microbiomes and to evaluate the utility of equine feces as a representative of the equine cecal

microbiome. Cecal and fecal samples were similar in Gram staining characteristics as the samples shifted from a Gram-negative dominated population prior to inulin-type fructan administration to a Gram-positive-dominated population after administration of fructan. The abundance of *Firmicutes* members was low and variable (0-30% of total bacteria) prior to fructan administration and *Firmicutes* members increased (>40% total bacteria) after fructan administration, in both cecal and fecal samples. The increase in *Firmicutes* was delayed in the fecal samples (8-20hr after fructan administration) relative to the cecal samples (4-12 hours after fructan administration). The abundance of *Streptococcus bovis/equinus*, a member of the *Firmicutes* phyla was similar between cecal and fecal samples prior to fructan administration and increased (>50% total bacteria) in both the cecum (2hr) and feces (8hr) after fructan administration. Gastrointestinal mean transit time, in horses fed forage, for solid and liquid fractions range from 23-52 and 18-31 hours, respectively (Cuddeford et al., 1995) with approximately 80% of the time is spent in the hindgut (Van Weyenberg et al., 2006). Therefore the change in the cecal microbiome as reported by Milinovich *et al*. (2007) was comparable to an anticipated 4-10 hour transit of forage from mouth to cecum (Van Weyenberg et al., 2006). The 4-6 hour lag between changes found in cecum and feces as reported by Milinovich *et al*. (2007) is shorter than expected based on an anticipated 14-42 hour transit of forage from cecum to feces (Van Weyenberg et al., 2006); however, the early changes in the feces may represent those occurring in the colon. Furthermore, the oligofructose administered by Milinovich *et al*. (2007) is a small molecule and was administered in a liquid medium and therefore would not be selectively retained in the cecum and ventral colon of the horse (Van Weyenberg et al., 2006) thus reducing transit time.

Simpson *et al*. (1999) used denaturing gradient gel electrophoresis (DGGE) to evaluate gut microbial similarity across the gastrointestinal tract of pigs and reported that rectal samples shared 13% similarity to cecal lumen samples, 19% similarity to proximal colon lumen samples, and 41% similarity to distal colon lumen samples. Fecal samples shared higher similarity with proximal (27%) and distal (79%) colon mucosal samples as compared to lumen samples. Hastie *et al*. (2008) did not detect a difference in the abundance of *Ruminococcus flavefaciens, Fibrobacter succinogenes,* and *Streptococcus bovis*, by use of real-time PCR, from the cecum to the feces. Although, the fecal microbiome may not be an exact representation of the cecal or colon microbiome, fecal samples are a non-invasive and indirect measurement of the hindgut microbiome of the horse and changes in the cecal and colon microbiome are reflected in feces.

2.8.3 - Nucleic Acid Extraction (Step 2)

Extraction of nucleic acids (DNA or RNA) from feces may be more challenging than extraction from animal tissues due to the complex nature of the fecal matrix. Numerous nucleic acid extraction protocols have been described for extraction of DNA for identity (Ariefdjohan et al., 2010; McOrist et al., 2002; Stauffer et al., 2008; Yu and Morrison, 2004; Zoetendal et al., 2006) or RNA for functional evaluation (Bera-Maillet et al., 2009). Commercial kit and more traditional non-kit protocols are available with their own benefits and pitfalls. Nucleic acid extraction kits have enhanced methods for removing DNA polymerase inhibitors (e.g. organic and phenolic compounds, and humic acids) (Juste et al., 2008; Wilson, 1997) although this benefit may come at the expense of reduced total nucleic acid recovery. Physical and enzymatic methods have been described to lyse cells prior to nucleic acid extraction. Gram-positive bacteria do not lyse with the same ease as Gram-negative bacteria, due to the thickness and density of their peptidoglycan cell wall layer (Cabeen and Jacobs-Wagner, 2005). Cuiv *et al*.(2011)

reported that a beading-based physical lysis extraction method was superior to a chemical/enzymatic method for Gram-positive (e.g. *Firmicutes* members) lysis. Due to the high abundance of *Firmicutes* (Gram-positive group) in the gut of horses (Daly et al., 2001) and other species (Suau et al., 1999; Tajima et al., 2000), a physical lysis approach seems most appropriate for evaluation of the gut microbime.

2.8.4 - Identifying a Target Gene (Step 3)

Microbial DNA and/or RNA sequences are commonly used for molecular evaluation of a complex microbial population. Therefore, selection of a target gene or genes in the population's genome or transcriptome is a key step in culture-independent evaluation. Genes targeted include those conserved across the whole bacterial population (present in all bacteria; i.e. 16S rRNA gene, *gyr*) or functional genes (genes encoding for enzymes present in only a subset of the population). Regions of a target gene can be conserved or variable. Conserved regions are portions of a gene sequence that are the same across different bacteria. Variable regions are portions of a gene sequence that differ between bacteria; variable regions allow for down-stream segregation of gene sequences from individual bacteria or groups of bacteria. Targeting of conserved regions of genes allow for evaluation of a complex bacterial community. Commonly targeted conserved genes include those within the ribosomal RNA operon (gene family) and housekeeping genes.

The 16S rRNA gene is common target for molecular techniques. The 16S ribosomal RNA (16S rRNA) gene is a component of the 30S ribosome and is approximately 1500 nucleotides in length. The intergenic spacer gene (IGS) is also found within the rRNA operon; the 16S rRNA and IGS genes serve as good target genes because they are highly conserved across bacteria. Additionally, the 16S and IGS genes harbor a variable sequence region that

allows for discrimination between bacteria. Other benefits of the RNA operon genes include multiple copy presence, which increases identification sensitivity. Also, there are multiple public databases, such as the Ribosomal Database Project (rdp.cme.msu.edu) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to increase the identification of unknown bacterial sequences. The 16S rRNA gene is a very stable molecule, both physically, due to its secondary structure (Garrett and Grisham, 1999; Olsen et al., 1986), and evolutionarily, as there is little change in the sequence over time. The latter is favorable because bacteria replicate rapidly and their genomes have frequent opportunity to change with each new generation.

Other conserved genes, such as RNA polymerase B (*rpoB*) (Dahllof et al., 2000), gyrase B (*gyrB*) (Yamamoto and Harayama, 1995), and elongation factor Tu (*tuf*) (Bera-Maillet et al., 2009), have been proposed as target genes (Juste et al., 2008). These genes are often found as single copies in a bacterial genome, which may be favorable since different 16S rRNA copies within the same bacterial genome may have different sequences (Vasquez et al., 2005). However, the limitation with these genes is that the databases are not as robust, which limits bacterial sequence identification, as for the 16S rRNA gene.

2.8.5 - Choosing a Technique to Study the Microbiome (Step 4)

Describing the Microbiome

Bacteria can be identified based on sequence similarity with previously identified sequences. The level of taxonomic identification: phylum (broad) > class > order > family > genus > species (narrow), is dependent on the nucleic acid sequence similarity to an identified sequence. For example, if an unknown sequence is 100% similar to the *Clostridium cellulolyticum* sequence then the unknown sequence is identified as *Clostridium cellulolyticum*. However, if the unknown sequence shares less than 100% sequence similarity with *Clostridium cellulolyticum* then the taxonomic classification is more broad (Table 1) (Acosta-Martinez et al.,

2008; Roesch et al., 2007). If the unknown sequence shares <75% similarity with *Clostridium cellulolyticum* then it is only classified at the kingdom level (bacteria) and thus could be a member of a different phyla.

Table 1 - Taxonomic assignment of unknown sequences using Clostridium cellulolyticum as an example.

Sequence similarity of unknown sequence with *Clostridium cellulolyticum*	**Level of taxonomic identification**	**Unknown sequence identification**
> 97%	Species (narrow)	*Clostridium cellulolyticum*
> 95%	Genus	*Clostridium*
> 92%	Family	*Clostridiaceae*
>91%	Order	*Clostridiales*
>85%	Class	*Clostridia*
>75%	Phylum	*Firmicutes*
>0%	Domain/ Kingdom (broad)	Bacteria

The number of members in a population represents bacterial abundance, which can be described in absolute or relative terms. Absolute abundance represents the total number of a given species or members of a taxonomic group (i.e. phylum) present per unit of sample (i.e. gram of feces). For example, the abundance of a specific bacterial phylum, such as *Firmicutes* may be expressed as $\log_{10}$ copies/g feces. Relative abundance represents the number of a given species or members of a taxonomic group (i.e. phylum) present relative to the total number of bacteria present in a sample. Therefore, data is presented as a percentage of total bacteria.

A bacterial population as a whole may be described based on its richness, which represents the number of different taxonomic groups in a population. Richness is typically

determined for sequences sharing 97% similarity (97% nucleotide similarity between bacterial sequences), which allows for species-level identification (Roesch et al., 2007). Therefore, a richness of 10 suggests the presence of 10 species. Bacterial diversity represents how many different taxonomic groups are present in a population and the abundance of each group. Bacterial population evenness is a measure of taxonomic distribution of a population. An even population is one that has equal representation of all bacterial groups (i.e. phyla). For example, a population containing 25% *Firmicutes*, 25% *Bacteroidetes*, 25% *Proteobacteria*, and 25% *Actinobacteria* is an even population. However, a population containing 80% *Firmicutes*, 10% *Bacteroidetes*, 5% *Proteobacteria*, and 5% *Actinobacteria* is an uneven population. The robustness of a bacterial population represents how resilient the bacterial population is to change (i.e. change in the host's diet).

Tools for use in Evaluating a Complex Microbial Community

A review of the numerous techniques available for evaluating complex microbial communities is provided in Table 2 and by Juste *et al*. (2008), Hall, Kelles and Blackall (2003), Milinovich *et al.* (2010), and Speknijder *et al*. (2001).

Table 2 - ***Review of molecular techniques for evaluating microbial communities.***

Method	Overview	Uses/Benefits	Limitations
Denaturing Gradient Gel Electrophoresis (DGGE)	Bacterial 16S rRNA gene sequences (or other target gene) are separated on a gel based on GC content (vs. size); sequences are visualized as bands on a gel with each band representing 16S rRNA gene sequences with similar GC content	• Monitoring microbial community changes • Evaluation of up to 12 samples in a single gel & 24 samples in a single run (2 gels/run) • No prior knowledge of the bacterial population needed • Low cost-per sample	• Data analysis dependent on end-point PCR products • Limited identification due to the small sequences used (200-700bp) • No spatial resolution • High up-front equipment cost • 2-day minimum turn-around • Standardization & Reproducibility • Co-migration of different sequences with similar GC content
Terminal-restriction Fragment Length Polymorphism (T-RFLP)	Bacterial 16S rRNA gene sequences (or other target gene) are fluorescently labeled, digested using enzymes targeting specific sequences, the separated based on size and detected using an automated sequencer; variation in DNA fragment lengths is positively associated with complexity/diversity of the bacterial community	• Monitoring microbial community changes • Sequence identification; large databases available (i.e. RDP, MiCA) to compare sequences to • High throughput automated sequence analysis	• Data analysis dependent on end-point PCR products • More than one restriction enzyme is needed to increase assay specificity • Diversity/population complexity overestimation can occur due to non-specific sequence fragmentation
Ribosomal Intergenic Spacer Analysis (RISA)	Bacterial ribosomal intergenic spacer (IGS) sequences are visualized as a pattern of bands on a gel; the bands represent IGS regions of varying lengths	• Fluorescently labeled primers can be tagged to the IGS sequences to enhance assay sensitivity • Automated if fluorescent tagging is used	• Data analysis dependent on end-point PCR products • Short IGS sequences can be overrepresented due to preferential PCR amplification prior to gel electrophoresis • One organism may be represented by IGS regions of different length

Clone library Generation	Single bacterial DNA sequences are incorporated into *E.coli* DNA and then sequenced to determine sequence identity	• Identification of individual bacteria • Relative abundance of a bacterial group can be calculated from total bacterial abundance	• Data analysis dependent on end-point PCR products • Time consuming & tedious • Need to produce thousands of clones to represent complex populations • Population bias as sequences with a low GC content may be preferentially cloned
Shotgun Sequencing	Cloning portions of a bacterial genome, sequencing, then piecing the genome pieces back together to establish a complete genomic sequence	• Identification of individual bacteria • Evaluation of multiple genes within a single bacteria	• Time consuming & tedious due to the need for clone library generation
High-throughput Sequencing	Sequencing of PCR amplified gene segments	• Identification of individual bacteria without the need to clone sequences; rapid turn-around • Provides information on thousands of bacterial sequences from each sample • Relative abundance	• Data based on end-point PCR products • Expensive sequencing instrumentation needed
Fluorescence *in situ* Hybridization (FISH)	Visualization of bacteria tagged using fluorescent probes	• Data is not biased by DNA extraction or PCR • Spatial resolution (location of bacteria within a cell or tissue section) • Detects presence/absence and estimates relative abundance of targeted bacteria • Can use flow cytometry (computerized) to analyze fluorescence in cells	• Prior knowledge of the bacterial population needed; • Less sensitive than PCR-based technologies for determining abundance
Real-Time PCR (Quantitative PCR [qPCR])	Quantification of bacteria by measuring the absolute or relative number of target genes (i.e. 16S rRNA gene or functional genes)	• Used to detect absolute abundance • High sensitivity • High specificity, particularly with TaqMan® technology	• Sensitivity and specificity are determined based on the complementation of selected primers to the target population • Specificity lower with SYBR® Green Technology vs. TaqMan® technology

Microbial Diversity

Methods for assessing microbial diversity include terminal restriction fragment length polymorphism (T-RFLP) (Tiquia, 2005), automated ribosomal intergenic spacer analysis (ARISA) (Sundset et al., 2009), and denaturing gradient gel electrophoresis (DGGE) (Endo et al., 2009; Hidetoshi et al., 2007; Milinovich et al., 2008; Muyzer, 1999; Muyzer et al., 1993; Muyzer and Smalla, 1998). These PCR-based methodologies are useful for assessing complex communities and do not require prior knowledge of the whole genome sequence of individual members.

In terminal restriction fragment length polymorphism, microbial communities are differentiated based on amplicon fragment length and sequence. Genes are amplified with fluorescently labeled primers, cut by a restriction enzyme, and then evaluated on an automated sequencer. Multiple restriction enzymes are used to enhance specificity of sequence identification. Sequence length determines community complexity. Sequences can be compared back to available databases, such as the RDP (http://rdp.cme.msu.edu) (Cole et al., 2009; Cole et al., 2005) for identification of the sequence.

Automated ribosomal intergenic spacer analysis (ARISA) targets the ribosomal intergenic spacer (IGS) region and differentiates communities by amplicon length. The IGS region provides greater variation in sequence and length (400-1200bp) (Juste et al., 2008; Popa et al., 2009) than the 16S rRNA gene, which allows for greater bacterial sequence segregation. Amplicons are produced using fluorescently labeled primers. The amplicon banding pattern is produced on an electrophoresis gel and visualized with a laser.

Denaturing gradient gel electrophoresis (DGGE) is a PCR-based technology where 16S rRNA gene fragments (200-700bp) are separated based on guanine + cytosine (GC) content

(Myers et al., 1985). Several samples (up to 24-32) may be run at a time; therefore, treatment within similar ecosystems or differences between ecosystems can be visualized in a single run. Limitations of PCR-DGGE include PCR bias (Juste et al., 2008), presence of multiple gene copies in a single bacteria cell (16S rRNA gene), co-migration of bands (similar GC content despite different sequence), limited classification depth for identification (due to short amplicon length), inability to quantify bacteria present, and limited standardization (Muyzer and Smalla, 1998). Temperature gradient gel electrophoresis (TGGE) is a similar technology where a denaturing chemical gradient is replaced with a temperature gradient. Single-strand conformation polymorphism (SSCP) is another similar methodology; however, this technology distinguishes community fingerprints by evaluating secondary structures formed after denaturation. The lack of GC-clamp primers in SSCP improves PCR performance.

Microbial Abundance

Real-time PCR is culture-independent method for evaluation of the abundance of specific microbes (Matsuki et al., 2004) or groups of microbes within a population (Fierer et al., 2005; Juste et al., 2008). This relatively inexpensive and rapid method may be used to target the 16S rRNA gene, or other target gene, for determination of bacterial abundance. The real-time PCR technology enables determination of initial sample DNA concentration in real time through detection of a fluorescent marker (i.e. SYBR® Green) (Guo et al., 2008b). Primer sets exist for studying specific groups within different communities; however, the bacterial members within each group or host can differ (Guo et al., 2008b; Ley et al., 2006b; Meier et al., 1999). Limitations of real-time PCR are low through-put nature (limited number of genes that may be quantified in a single run) and technical skill required. In addition to the effect of PCR inhibitors (Juste et al., 2008; Wilson, 1997) on DNA amplification, other factors that influence

amplification efficiency include GC content (negative correlation) (Dutton et al., 1993; Reysenbach et al., 1992), genome size and target gene (i.e. 16S rRNA) copy number (Farrelly et al., 1995; Kanagawa, 2003).

Microbial Identification

Methods for bacterial identification include fluorescent *in situ* hybridization (FISH), sequencing of clone libraries (Daly et al., 2001), conventional and whole-genome shotgun sequencing (Juste et al., 2008), and high-throughput sequencing platforms (e.g. pyrosequencing) (Andersson et al., 2008). Identification can be carried out by sequencing DNA present in gel-based methodologies (i.e. DGGE); however, DNA fragments are of limited size and bands rarely represent a single microbial member.

Fluorescent *in situ* hybridization (FISH) is an identification methodology that relies on binding of labeled fluorescent probes to specific intact bacteria present in tissue sections (i.e. intestinal mucosa) or on free bacterial cells via flow cytometry (Juste et al., 2008). Therefore, bacterial are evaluated without the need for DNA extraction and PCR and thus bacterial abundance is not altered by DNA amplification. Probe hybridization (Daly et al., 2011; Daly and Shirazi-Beechey, 2003) has been used to determine bacterial abundance. However, probe hybridization is less sensitive at detecting individual bacteria than real-time PCR and thus less appropriate for analysis of abundance (Farrelly et al., 1995; Juste et al., 2008). Fluorescent *in situ* hybridization is ideal for detecting presence/absence of bacteria and for spatial resolution (location of bacteria within a cell or tissue section) and would be an excellent method for tissue samples, such in the evaluation of mucosal adherent bacteria. However, a limitation of the Milinovich *et al*.(2007) study is that FISH was applied to detect bacterial abundance.

Furthermore, FISH was used to evaluate cecal fluid and feces, as opposed to cecal mucosal sections, where the location of microbes is likely of little clinical interest.

Analysis of clone libraries is a time-consuming process where unknown DNA amplicons are spliced into an *E.coli* plasmid designed such that (1) the *E.coli* cells without the plasmid will not survive and (2) *E.coli* cells containing a plasmid without the unknown sequence will be phenotypically distinct from *E.coli* cells containing the plasmid with the unknown sequence (Juste et al., 2008) (Appendix M). Once *E.coli* cells with the target phenotype are cultivated and harvested, DNA is extracted and the sequence, minus the *E.coli* plasmid sequence is used to identify the unknown bacteria present in the target sample. Therefore, single community member identification is possible. Limitations of this technology, aside from time, include the need to harvest multiple *E.coli* colonies to ensure a representation of the unknown sequences are made and reduced efficiency of high GC sequence insertion into the *E.coli* plasmid. Clone library generation (Daly et al., 2001; Willing et al., 2009; Yamano et al., 2008) has been utilized to measure the abundance of equine hindgut bacteria. Clone-library sequencing is performed with end-point PCR amplicons (i.e. 16S rRNA gene amplicons) present in the target community (i.e. gut or feces) and thus do not accurately capture bacterial abundance because quantification is performed after DNA is amplified.

High-throughput sequencing technology, such as pyrosequencing, enables identification of multiple organisms at once. Pyrosequencing is sensitive tool to characterize the richness of a complex bacterial community and identify thousands of bacteria present (Trochimchuk et al., 2003). Pyrosequencing provides a rapid turn-around with increased instrument-controlled steps (Juste et al., 2008; Turnbaugh et al., 2006) and is a more sensitive method for evaluating bacterial identification than clone library generation (Bent and Forney, 2008). High-throughput

sequencing methods such as pyrosequencing are utilized for quantification of microbial abundance in human feces (Andersson et al., 2008; Dethlefsen et al., 2008). However, sequencing methods are expensive and, as with clone libraries, abundance is determined after DNA is amplified.

Regardless of the method for bacterial identification (clone libraries vs. high-throughput sequencing), the assay is limited by the database of known bacterial sequences, which is constantly growing.

2.9 - Equine Hindgut Microbes: What We Know Today

2.9.1 - Equine Hindgut Microbes in Healthy Horses

Hindgut microbes enable the horse to thrive on a forage-based diet, yet the equine hindgut microbiome has received little attention compared to the rumen microbiome of cattle. Reports of the equine fecal and gut microbiome can be challenging to compare due to differences in diets between studies and the lack of standardization of molecular techniques between studies.

Daly *et al*. (2001) evaluated the hindgut microbiome of slaughtered healthy horses; horses were fed grass prior to slaughter. The hindgut microbiome was dominated by *Firmicutes* (72% of coned sequences) and *Bacteroidetes* (20%) phyla members; other phyla represent only 8% of cloned sequences. Over one third of sequences obtained from the equine hindgut were affiliated with *Clostridia*, a class within the *Firmicutes* phylum cluster XIVa, which is a prominent group in human feces (Daly et al., 2001; Suau et al., 1999). The *Firmicutes* phylum is a functionally diverse group in the horse as *Firmicutes* members range from saccharolytic (Julliand et al., 1999) to fibrolytic (Hastie et al., 2008) species. Willing *et al*. (2009) compared the fecal microbiome of horses fed two different diets (cool-season grass haylage versus cool-season grass hay plus high starch concentrate) by use of both culture-dependent and independent

(T-RFLP with subsequent clone library sequencing) methodologies. Fecal communities were dominated by members of the *Firmicutes* and *Bacteroidetes* phyla and represented 73% and 27% of T-RFLP clones, respectively, in horses fed the haylage plus high starch concentrate which is similar to reports from Daly *et al*. (2001). However, when Willing *et al*. (2009) fed the same group of horses an all forage diet, *Firmicutes* and *Bacteroidetes* members represented 46% and 49% of clones, respectively. The inverse ratio of *Firmicutes: Bacteroidetes* is unique as the *Firmicutes* phylum dominates the gut communities of multiple species (Eckburg et al., 2005; Guo et al., 2008a; Leser et al., 2002; Ley et al., 2005). Second, the discrepancy between the abundance of *Firmicutes* and *Bacteroidetes* in horses fed a forage diet in the Willing *et al*. (2009) vs. Daly *et al*. (2001) study is quite large and may reflect an effect of molecular methodology used. Sequence lengths in T-RFLP are determined by both primers used to amplify target bacterial DNA and by amplicon digestion with an enzyme. However, with clone library generation, sequence length is determined only by primers used to amplify target bacterial DNA. Therefore, sequence length and identification method may have caused the discrepancy between the Willing *et al*. (2009) and Daly *et al*. (2001) studies.

The equine hindgut microbiome is dominated by fibrolytic bacteria according to both culture-based (Garner et al., 1975; Mackie and Wilkins, 1988) and culture-independent studies (Hastie et al., 2008; Lin and Stahl, 1995). Fibrolytic bacteria are represented in both the *Firmicutes* and *Bacteroidetes* phyla (Gibson and Roberfroid, 1995). The fibrolytic bacterial species extensively studied in the rumen are *Fibrobacter succinogenes*, a member of the *Fibrobacteres* phyla, and *Ruminococcus flavefaciens* and *Ruminococcus albus*, members of the *Firmicutes* phyla (Denman and McSweeney, 2006; Tajima et al., 2001; Wang et al., 1997). Of these, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* represent 12% and 4%,

respectively, of total hindgut bacteria (Hastie et al., 2008; Julliand et al., 1999; Lin and Stahl, 1995). However, the abundance of *Fibrobacter* spp. in the equine gut appears to vary by molecular tool used and region of the gut evaluated. For example, *Fibrobacter* spp. represented 0.01% of total hindgut bacteria (Daly et al., 2001) by use of clone library generation; 1.2% of cecal bacteria as reported by Julliand *et al*.(1999), and 4% and 12% in the colon and cecum, respectively, by use of probe hybridization (Daly and Shirazi-Beechey, 2003; Lin and Stahl, 1995). Other fibrolytic *Firmicutes* include *Eubacterium* spp., and *Clostridiaceae*, which have been described in the horse (Daly et al., 2001).

2.9.2 - Effect of Diet on the Equine Hindgut Microbiome

de Fombelle *et al*. (2003) utilized culture-dependent methods to evaluate the gut microbiome of horses fed high fiber feed plus straw vs. high starch feed plus hay. Anaerobic bacteria were cultured from all regions of the gut, the concentration of cellulolytic bacteria was higher in the hindgut than foregut, and cellulolytic bacteria were lower in the cecum and left dorsal colon of horses fed the high starch feed plus hay diet (de Fombelle et al., 2003). Similarly, Julliand *et al.* (2001) reported that concentration of cellulolytic bacteria and acetate production was lower in the cecum and colon of horses fed hay plus barley versus horses fed hay alone. Muhonen *et al*.(2009) evaluated the effect of sudden diet change from hay to silage or haylage on the culturable microbial profile in adult horses. No changes in microbial counts or VFA production was noted in the first 28 hours. A statistical increase in *Lactobacilli* spp. and decrease in *Streptococci* spp. did not occur until 21 days after diet change.

Although, diet appears to influence the equine hindgut microbiome, changes in the equine hindgut microbiome do not always result in disease. For example, Willing *et al*.(2009) reported that the abundance of *Lactobacilli* spp. and *Streptococci bovis*, members of the *Firmicutes* phyla,

were higher in feces of the healthy horses fed the hay plus high starch feed as compared to the horses fed haylage.

2.9.3 - Equine Gut Microbes In Disease

In the horse, abnormal shifts in equine hindgut bacteria are associated with colic, diarrhea, and laminitis (Al Jassim and Andrews, 2009; Bailey et al., 2004; Milinovich et al., 2008; Milinovich et al., 2007; Milinovich et al., 2006; Rowe et al., 1994). Multiple authors have characterized the affect of rapidly fermented carbohydrates on the hindgut microbiome by use of culture-based (de Fombelle et al., 2003; Garner et al., 1975; Garner et al., 1978; Respondek et al., 2008) and culture-independent (Al Jassim et al., 2005; Milinovich et al., 2008; Milinovich et al., 2007; Milinovich et al., 2006) methods. The hindgut microbial community responded to rapidly fermentable carbohydrates (sugar, starch, fructan) by increasing the production of lactate (Julliand et al., 2001; Medina et al., 2002) and shifting from a Gram-negative dominated population to a Gram-positive dominated population associated with an increased abundance of *Streptococcus* spp. (Garner et al., 1975; Garner et al., 1978; Milinovich et al., 2008). Dietary starch (Garner et al., 1978; Garner et al., 1977) and inulin-type fructan (Bailey et al., 2007; Kalck et al., 2009; Milinovich et al., 2006; van Eps and Pollitt, 2006) have been used to experimentally induce laminitis. The fructan-overload laminitis model has been associated with decreased cecal pH (Milinovich et al., 2007), increased abundance of cecal Gram-positive *Firmicutes* bacterial phylum members, increased (Milinovich et al., 2008), alterations in systemic vascular and inflammatory mediators (Bailey et al., 2009), and lamellar inflammation (Visser and Pollitt, 2011), each associated with alterations in lamellar architecture and subsequently laminitis (de Laat et al., 2011).

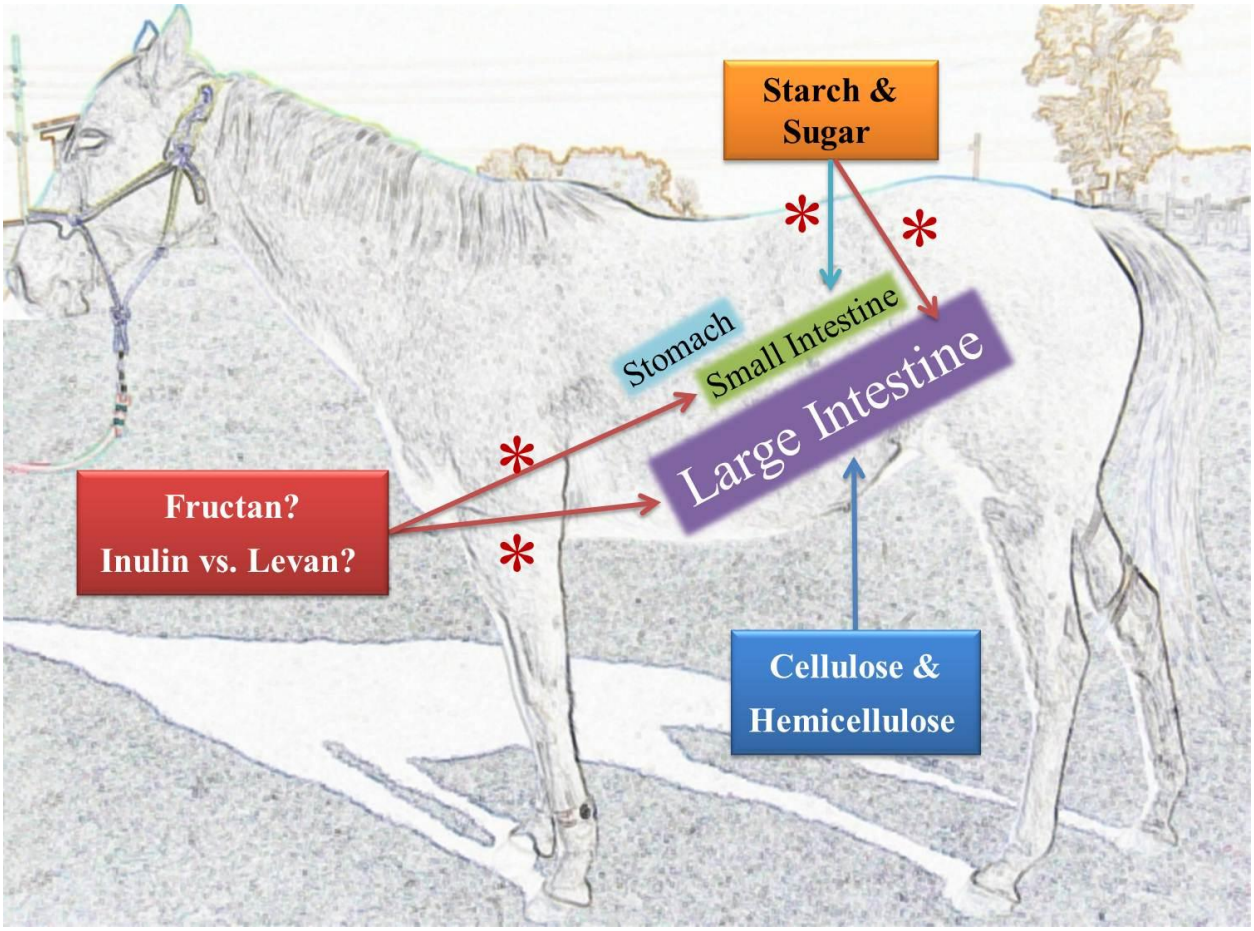
2.10 - Summary

The horse is able to thrive on a high fiber diet due to the millions of microbes, mostly bacteria, which reside in the hindgut. Furthermore, horses can become overweight and obese on forage alone. The equine hindgut microbiome may be associated with equine obesity such that obese horses may have a hindgut microbiome capable of extracting more calories from the horse's high fiber diet. An obese microbiome in the horse could explain, at least in part, why some horses maintain an obese phenotype on the same forage-based diets as lean counterparts.

Obesity and feeding high NSC diets increases a horse's risk of developing insulin resistance and subsequently laminitis. Administering NSC boluses alters the equine hindgut microbiome and induces laminitis; grass consumption has been associated with laminitis laminitis and this association may be due to the NSC in grasses.

Despite the reliance of the horse on its hindgut microbes and the adverse effects of disturbances in the equine hindgut microbes, the influence of cool-season grass NSC (as a source of levan-type fructans) on gut microbes and subsequently equine health has not been fully elucidated (Figure 2). There is still much work to be done before the scientific community understands the complexity of the equine hindgut microbiome and how it can be implemented in maintenance of host health and potentially disease treatment, such as obesity.

Figure 2 - ***Location of carbohydrate digestion and fermentation in the equine gut.***



*Blue arrows normal physiology; Red arrow represents pathways where carbohydrate are rapidly fermented in the equine hindgut; * indicates pathways that may be linked to host illness (i.e. laminitis)*

Section 3 - Effects of High and Moderate Non-Structural Carbohydrate Hay on Insulin, Glucose, Triglyceride, and Leptin Concentrations in Overweight Arabian Geldings

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3.1 - Summary

The objective of this study was to determine the effects of high and moderate non-structural carbohydrates (NSC) hay on insulin, glucose, triglyceride, and leptin concentrations in overweight Arabian geldings. Eight adult overweight (average BCS 7 [9 point scale]) Arabian geldings were fed each of two orchardgrass hays, high NSC (18% DM) and moderate NSC (12% DM), in a cross over design during two 28-day periods. Body weight and body condition score assessment along with blood sampling to measure insulin, glucose, leptin, and triglyceride concentrations were performed on days 0, 7, 14, 21, and 28 of each period. Effects of hay, period, day, and day*hay on plasma glucose and serum leptin were not detected. Serum insulin was influenced by hay ($p=0.001$), day ($p=0.03$), and day*hay ($p=0.04$). Insulin concentrations were higher only on day 7 in the high NSC group (15.6µIU/ml) than the moderate NSC group (9.5µIU/ml), but not by day 14 ($p=0.0007$). Plasma triglyceride was influenced by period

(p=0.0003), day*period (p<0.0001), and day*hay (p=0.02). Hyperinsulinemia was not observed in the overweight Arabian geldings fed either a moderate or high NSC hay.

3.2 - Introduction

Obesity and insulin resistance are important problems facing the equine industry today due to their association with an increased risk for development of laminitis, an extremely painful and debilitating condition of the equine hoof. Increased adiposity is positively associated with the development of insulin resistance in horses (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007). Thatcher *et al*. (2008) and Geor *et al*. (2007) conducted a prospective study of 300 horses in Southwest Virginia and found that 54% of the horses were overweight or obese, and 10% of the horses were hyperinsulinemic. A strong positive association between hyperinsulinemia, an indicator of insulin resistance, and adiposity was determined, as 18% of the overweight or obese horses were hyperinsulinemic versus only 1.4% of the lean horses.

Insulin resistance has also been positively associated with consumption of diets that contain high concentrations of enzymatically digestible carbohydrates (Treiber et al., 2005a). Therefore, restriction in enzymatically digestible carbohydrates, such as those found in grains (i.e. starches and sugars), is often recommended in the management and prevention of laminitis. While, the risk of excess enzymatically digestible carbohydrate consumption in the form of grains is well recognized, the risk of carbohydrates from forage is less clear.

Forages contain high concentrations of structural carbohydrates (cellulose and hemicellulose) that are fermented in the large intestine by microbes. Forages also contain additional carbohydrates that include mono/disaccharides, starches, and fructans. Nomenclature of carbohydrates in forages is complex as they can be classified by physiologic characteristics (enzymatically digestible or fermentable) or by analytical characteristics (solubility in water or

ethanol, direct starch measurement). Non-structural carbohydrates (NSC) are an analytical estimate of soluble carbohydrates in forages. Fructans, a group of non-structural fibers, are not directly measured on routine forage analysis and are components of the NSC fraction along with mono/disaccharides and starches. Fructans can accumulate in high concentrations in cool-season grasses and are proposed to be a risk factor for laminitis (Longland and Byrd, 2006).

Horse owners can be challenged with the management of obese and overweight horses with respect to forage in their diet. Fifty percent of laminitis cases are associated with the consumption of fresh forage (USDA, 2000). Unique carbohydrate components of cool-season grasses, specifically fructans, are rapidly fermented in the hindgut of the horse and may trigger laminitis (Kalck et al., 2009; van Eps and Pollitt, 2006). Likewise, pre-cecal fermentation of fructans in the horse may influence insulinemic and glycemic responses to a forage meal (Bailey et al., 2007; Coenen et al., 2006). Characterization of the carbohydrate profile of forages, either specifically as simple sugars, starches, and fructans or collectively as non-structural carbohydrates (NSC), may allow for identification of forages suitable for feeding horses with or predisposed to laminitis.

Although obesity, insulin resistance, and laminitis are observed in pastured ponies (Carter et al., 2009b; Treiber et al., 2006), the role of forage carbohydrates in these disorders is poorly understood. The term Equine Metabolic Syndrome (EMS) has been coined to collectively describe overweight and insulin resistant horses with clinical or subclinical laminitis (Johnson, 2002). The recent American College of Veterinary Internal Medicine EMS consensus statement suggested that horses with EMS should be fed hay with a NSC concentration <10% on a dry matter basis (DMB) (Frank et al., 2010). This recommendation requires further investigation as

there has been little exploration into the effect of cool-season grass NSC, with respect to appropriate concentrations to feed to horses at risk of laminitis .

The objective of this study was to determine the effects of feeding moderate and high NSC hay on insulin, glucose, triglyceride, and leptin concentrations along with physical parameters in overweight arabian geldings. We hypothesized that geldings fed high NSC hay would have higher fasted serum insulin and plasma glucose concentrations than horses fed moderate NSC hay.

3.3 - Materials and Methods

3.3.1 - Animals and Study Design

Eight overweight (mean BCS 7 $\pm$ 1 [9 point scale], mean body wt 488 $\pm$ 65 kg, age mean 16 $\pm$ 4 years), Arabian geldings were alloted to the study. Geldings were maintained on a 0.9 hectare pasture for 30 days prior to the initiation of the study and were randomly assigned to one of two hays fed for a 28 days (period 1). A washout period was then applied for 28 days on the previous 0.9 hectare pasture after which geldings were fed the alternate diet for 28 days (period 2). During periods 1 and 2, geldings were housed and fed in individual box stalls bedded with pine shavings; geldings had access to individual dry paddocks. Attitude, digital pulses, hoof warmth, and soundness were monitored and recorded twice daily in all geldings during periods 1 and 2. Except for short walks in-hand to assess soundness daily, geldings received no exercise during periods 1 and 2. Geldings were dewormed once with Fenbendazole (Panacur® 10% suspension, Intervet, Millsboro, DE) PO during the acclimation period and Ivermectin (Ivomec® 1% suspension, Merial, Duluth, GA) PO during the washout period. The study was conducted from November 2008 through February 2009 at the Virginia-Maryland Regional College of

Veterinary Medicine. All procedures were approved by Virginia Tech's Institutional Animal Care and Use Committee (Protocol 08-217-CVM).

3.3.2 - Diet

Geldings had free choice access to 0.9 hectare of a mixed cool-season grass (fescue, kentucky bluegrass, orchardgrass) pasture and a vitamin-mineral supplement (EquiMin® Granular, Southern States Cooperative, Richmond, VA; Appendix C) during the 30 day acclimation period. Pasture forage was sampled at 20 randomly generated GPS sites (ESRI ArcGIS® software and Garmin GPSmap 60CS). Forage was collected from a 0.3x0.3 meter square, 5.1cm above the ground into paper bags then dried in a 55ºC drying oven (Precision Freas Mechanical Convection Ovens Model 645, Pacific Combustion, Torrance, CA) for 72 hours to achieve <10% moisture. During the washout period geldings were offered free choice access to a cool-season grass/alfalfa (<5% alfalfa) mix hay and a vitamin-mineral supplement. The washout hay was sampled 7 days prior to initiation of period 2. For periods 1 and 2, investigators selected a hay with moderate NSC content to represent average commercially available grass hay and a high NSC hay to represent grass at an immature state. Baled hay was chopped in a round-bale chopper (New Holland Forage Chopper, New Holland, PA) to a 5cm length chop to reduce variation between bales. Approximately 114g of the high and moderate NSC hays were randomly sampled daily during periods 1 and 2 to create weekly sub-samples of each hay. All dry forage samples were stored in plastic bags at room temperature and were sent to a commercial forage laboratory (Equi-Analytical, Ithaca, NY) for nutritional analysis (Table 3) at the completion of the study.

Table 3 – Average nutrient analysis of forages fed to geldings during the study and grass hay analysis from a commercial laboratory[a].

Item	High NSC hay[b]	Moderate NSC hay[b]	Acclimation Pasture[c]	Washout Hay[c]	Equi-Analytical Reference interval[a]
Dry Matter (%DM)	90.3 ± 0.6	91.6 ± 0.4	93.4	91.1	90.6 - 93.3
DE (Mcal/kg DM)	2.25 ± 0.05	2.09 ± 0.05	1.91	2.06	1.8 - 2.2
CP (%DM)	19.3 ± 1.1	14.0 ± 0.6	11.7	13.1	6.9 – 14.7
ADF (%DM)	30.0 ±1.0	35.0 ± 1.6	40.7	38.5	34.4 – 43.6
NDF (%DM)	53.0 ± 2.7	60.6 ± 1.7	69.9	62.1	56.5 – 69.9
WSC (%DM)	16.7 ± 2.0	10.5 ± 0.8	6.3	8.6	6.5 – 15.2
ESC (%DM)	11.4 ± 2.8	7.4 ± 1.4	5.0	5.9	4.6 – 10.3
Starch (%DM)	1.2 ± 0.2	1.0 ± 0.4	0.7	1.2	0.9 – 3.7
NSC (%DM)	17.9 ± 2.0	11.5 ± 0.8	7.0	9.8	8.0 – 17.7
Ca (%DM)	0.32 ± 0.04	0.48 ± 0.03	0.46	0.49	0.3 – 0.7
P (%DM)	0.30 ± 0.02	0.40 ± 0.01	0.23	0.33	0.2 – 0.3

[a] *Equi-Analytical Laboratories (Ithaca, NY); range is mean +/- 1 SD for 10,000 - 40,000 samples based on analyte*

[b] *Values include mean ± SD (n=8)*

[c] *Values include a single measurement on a composite sample.*

Digestible energy (DE) was estimated by calculation; DE (kcal/kgDM=2,118+12.18(CP%)-9.37(ADF%)-3.83(hemicelluloses%)+47.18(fat%)+20.35(NSC)-26.3(ash%)(R^2-0.88) (Pagan, 1998). Crude protein (CP) was measured by use of kjeldahl method (AOAC 984.13), acid detergent fiber (ADF) and neutral detergent fiber (NDF) measured by use of Ankom A200 Filter Bag Technique (Ankom Technology, Macedon, NY), water (WSC) and ethanol soluble carbohydrates (ESC) (Hall et al., 1999), starch measured by use of YSI 2700 Select Biochemistry Analyzer (YSI Inc Life Sciences, Yellow Springs, OH), calcium and phosphorus were measured by use of acid digestion and spectrophotometry (CEM, Matthews, NC). Values for non-structural carbohydrates (NSC) were calculated using the sum of WSC and starch; NSC was not directly measured using chemical analysis. Within period coefficients of variation for NSC concentrations of the high and moderate NSC hays were 11% and 7% respectively.

Digestible energy requirements were initially calculated for each horse based adult average maintenance (NRC, 2007) using September body weights and initial forage analysis of the high and moderate NSC hays. During each period geldings were fed to maintain September body weights. Total daily hay was divided into two equal portions and fed at 0800 and 1600 hours. The hay ration was supplemented with the vitamin-mineral supplement (mean 71 grams/day) to meet requirements for adult maintenance (Gro-Trac® Elite, Kentucky Equine Research, Versailles, KY). Hay present the following morning was weighed to calculate total daily intake. Water was provided free choice via automatic waterers throughout the study.

3.3.3 - Sampling

Geldings were weighed, condition scored (Henneke et al., 1983) and venous blood samples were collected from the jugular vein on days 0, 7, 14, 21, and 28 of each period. Body weight was measured using a calibrated horse weight tape throughout the study and additionally

by digital scale (Cambridge Scale Works, Honey Brook, PA) on days 0 and 28 of each period. Body condition score was assessed by the same investigator throughout the study. Day 0 blood samples were drawn before geldings were moved into stalls; all other samplings were performed prior to the morning feeding. Blood was collected into 10ml tubes (BD Vacutainer®, Franklin Lakes, NJ) containing EDTA for triglyceride analysis, lithium heparin for glucose analysis, and no anticoagulant for insulin and leptin analyses. Plasma from EDTA and lithium heparin tubes was harvested within 30 minutes of collection after centrifugation (3,000 x g). Serum from no additive tubes was harvested after storage at 22°C for 3 hours and centrifugation (3,000 x g). Plasma and serum samples were stored at -80°C until analysis.

Analysis of samples

Plasma glucose and triglyceride concentrations were measured in quadruplicate and duplicate respectively by use of an enzymatic spectrophotometric method (Synchron® Systems Multi Calibrator, Beckman Coulter, Brea, CA). Serum insulin (Coat-A-Count® Insulin, Siemens Medical Solutions Diagnostics, Deerfield, IL) and leptin (Multi-Species Leptin RIA Kit, Linco Research, St.Charles, MO) concentrations were measured in duplicate by use of a commercial radioimmunoassay, both validated for use in the horse (Cartmill et al., 2003; Freestone et al., 1991). Intra-assay coefficients of variation were 1.6%, 1.1%, 2.0%, and 2.8% for insulin, glucose, triglyceride, and leptin, respectively.

3.3.4 - Statistical analysis

Statistical analysis was performed using repeated measures ANOVA followed by Tukey's procedure for multiple comparisons. For each outcome, the linear statistical model included hay type, day, period, horse, period*day, and hay*day as fixed effects. The model also specified that measurements were repeated over each horse within a period. Interactions

between hay and day were evaluated using the slice option of the GLIMMIX procedure. Plasma triglyceride concentrations were influenced by period (p=0.0003); therefore, triglyceride data were analysed within period. Effects of Hay, Period, and the interaction between Hay and Period on day 0 body weight, day 28 body weight, body weight change, intake as a % offered and as kg/horse/day, along with DE and fructans consumption were assessed using mixed model ANOVA followed by Tukey's procedure for multiple comparisons. This model also included Horse ID as a random effect. For each model, residuals plots were inspected to verify the assumption that the errors followed a normal distribution with a constant variance. Statistical significance was set at alpha=0.05. All analyses were performed using SAS software (SAS® Software version 9.2, Cary, NC).

3.4 - Results

Body weight (Table 4) was influenced by period (p=0.0002), day (p=0.0002), and day*period (p=0.0023). Geldings lost weight during period 1 and maintained weight during period 2. Daily hay fed was adjusted upward for period 2 due to the weight loss during period 1 to meet the goal of maintaining September body weights and minimize the effects of body weight and BCS changes on blood parameters. Digital pulses and hoof warmth remained unremarkable through the study and lameness was not detected in the geldings.

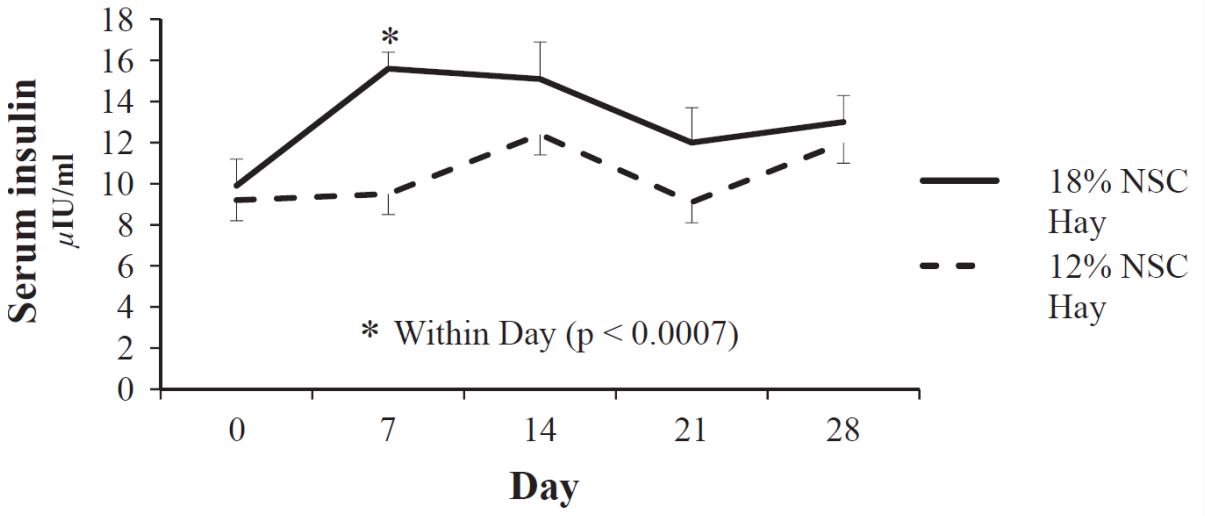
Table 4 - *Arabian Gelding parameters collected during each period of the study.*

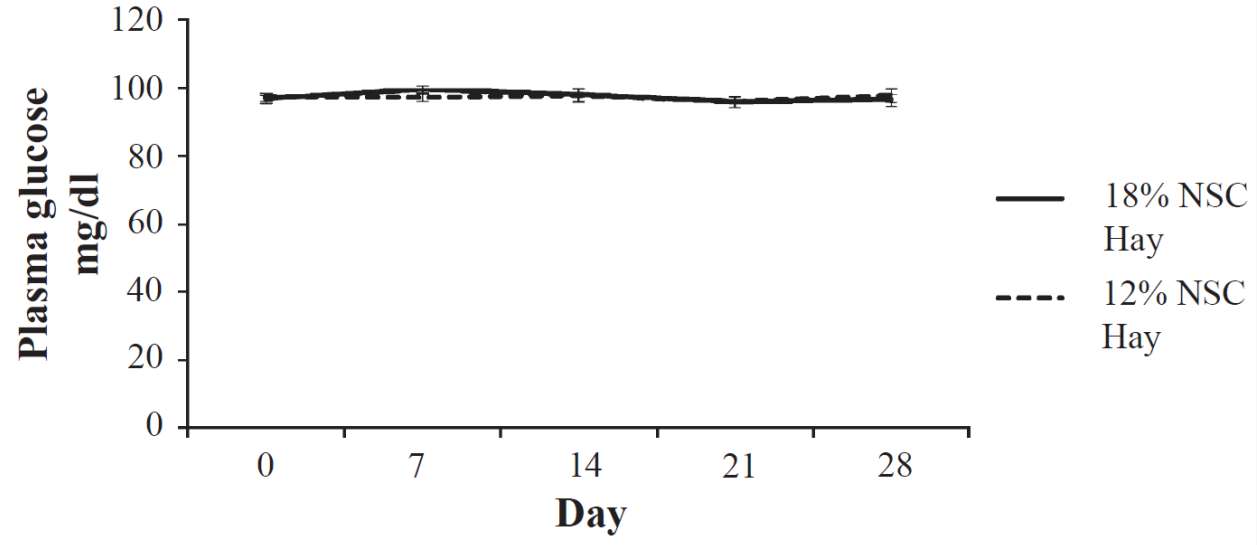
Period	Mean BCS (1-9/9)	BW Day 0 (kg)	BW Day 28 (kg)	BW change (kg)	Intake (% offered)	Consumption (kcal DE/kg BW)
1	7 ± 1.5 [a]	484 ± 57 [a]	471 ± 54 [b]	-13 ± 7 [a]	99 ± 1.6 [a]	32.1 ± 1.2 [a]
2	7.3 ± 1.3 [a]	471 ± 55 [b]	469 ± 53 [b]	-2 ± 5 [b]	97 ± 3.1 [a]	34.3 ± 2.0 [b]

Values are Mean ± SD; BW = Body weight (scale); DE = Digestible energy. Values with different superscripts within a column differ (p<0.05).

Effects of hay, day, and day*hay on plasma glucose, serum leptin (Figure 3), and body weights were not detected (p>0.05). Serum insulin was influenced by hay (p=0.001), day (p=0.03), and hay*day (p=0.04). Insulin concentrations (Figure 3) were higher on day 7 in the high NSC group (15.6µIU/ml ± 0.8) than the moderate NSC group (9.5µIU/ml ± 0.8). Plasma triglyceride was influenced by period (p=0.0003), day*period (p<0.0001), and day*hay (p=0.02). On day 0 triglyceride concentrations (Figure 4) were higher (p=0.0002) in period 2 (49.1mg/dl ± 5.1) than period 1 (24.2mg/dl ± 5.1). Plasma triglyceride concentrations were influenced by day (p=0.0001, p=0.0083) during period 1 and 2 respectively. From day 0 to 28, triglyceride concentrations increased during period 1 and decreased during period 2.

Figure 3 - ***Insulin, glucose, and leptin concentrations (mean ± SEM) on each sampling day in Arabian Geldings fed a High (18% DM) and Moderate (12% DM) Non-Structural Carbohydrate (NSC) Hay.***





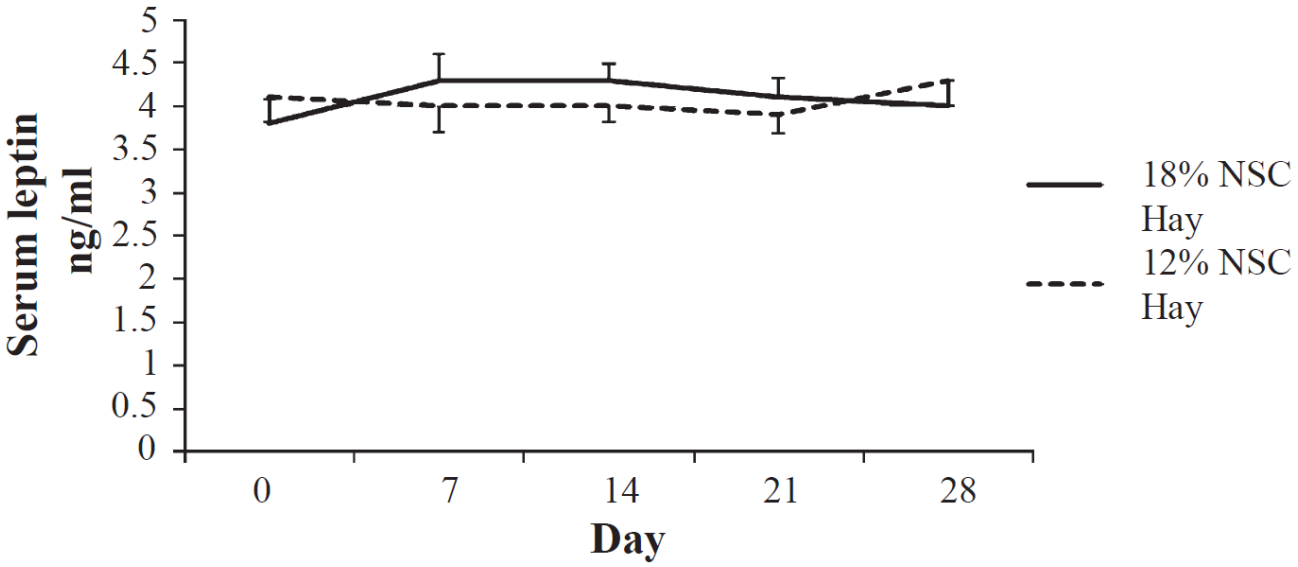
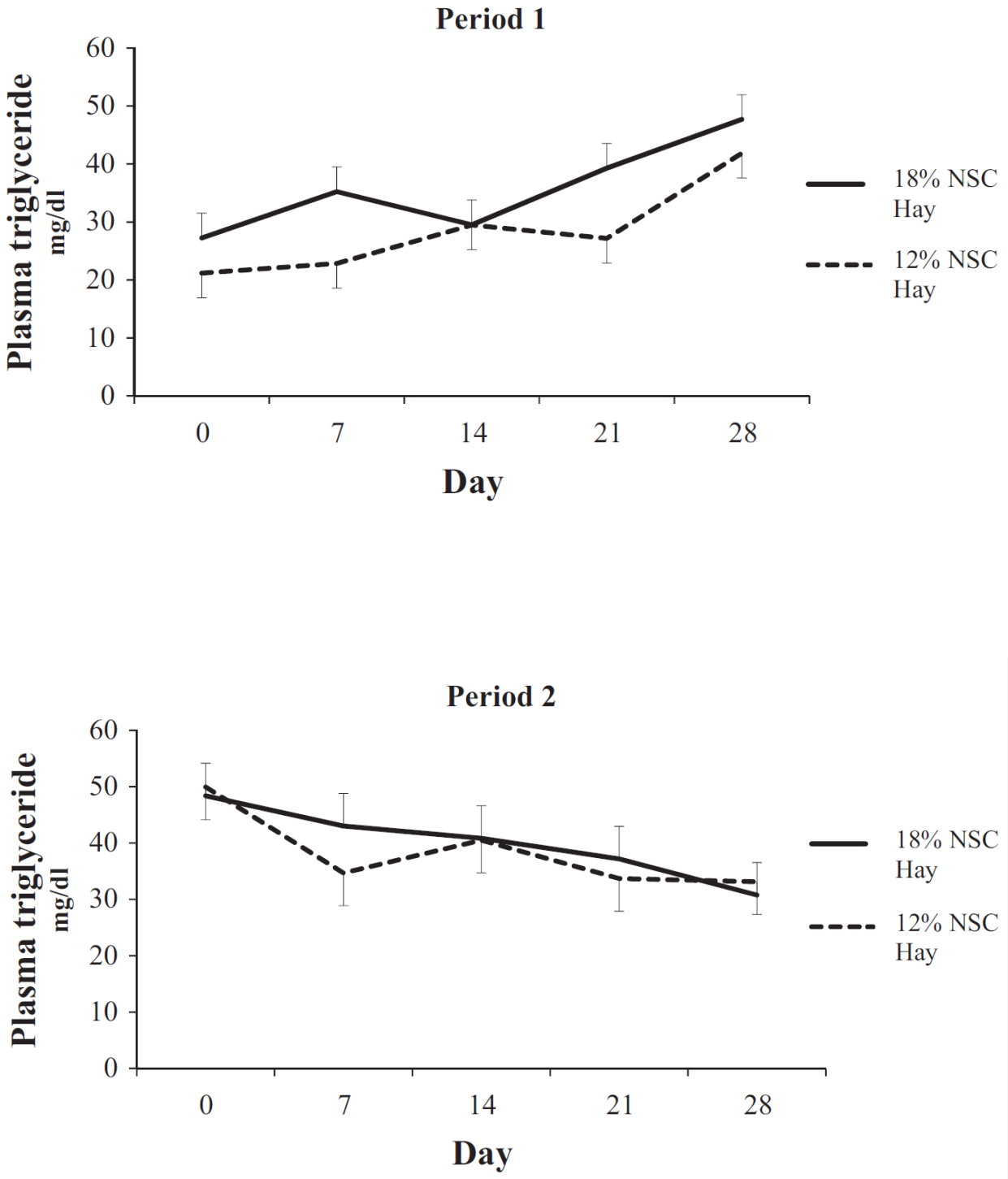

Serum leptin ng/ml
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
7
14
21
28
Day
18% NSC Hay
12% NSC Hay

Figure 4 - ***Triglyceride concentrations (mean +/- SEM) on each sampling day, within period, in Arabian Geldings fed a High (18% DM) and Moderate (12% DM) Non-Structural Carbohydrate (NSC) Hay.***



3.5 - Discussion

The EMS consensus statement pannel recommends feeding a hay with <10% NSC DMB for EMS horses to reduce the glycemic and insulinemic responses to a meal (Frank et al., 2010). However, the effect of variable NSC concentrations in hay on insulin and glucose concentrations in horses has not been evaluated prior to this study to the authors' knowledge.

Horses with increased adiposity are at increased risk of developing insulin resistance (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007) and laminitis; therefore, the authors of this study chose to use overweight horses. High (target ≥18% NSC) and moderate (target 10% NSC) grass hays were solicited for the study. The study hays were classified as high and moderate NSC based on commercial forage test laboratory reference data (Table 3). Starch concentration was relatively similar between the two study hays. The difference in NSC content was primarily mono/disaccharides and fructan oligo/polysaccharides.

Enzymatically digestible carbohydrates directly influence serum insulin in horses as they are digested and absorbed in the small intestine (Jose-Cunilleras et al., 2004). The role of fructans on insulin dynamics is less clear as the monosaccharide subunits are linked by *beta*-glycosidic bonds, which are not degraded by mamallian enzymes (Longland and Byrd, 2006). Fructans are rapidly fermented by microbes in the equine cecum and large colon (James et al., 2003; Niness, 1999). However, some authors have proposed that fructans may also be fermented prior to the large intestine (Bailey et al., 2007; Coenen et al., 2006).

The total estimated fructan concentration consumed from either study hay per kg body weight per day (Table 5) was below the low dose (5g fructan/kg body weight) associated with experimental induction of laminitis or an increase in insulin concentrations (Bailey et al., 2007; Kalck et al., 2009). Inulin, the fructan used to induce laminitis, has a lower degree of

polymerization than the fructans commonly found in forages. Caution may be warranted in assuming that the biological effects of inulin may be applicable to all fructans. Crude protein concentration was higher in the high NSC hay as compared to the moderate NSC, but the authors are unaware of any relationship between dietary protein and insulin response in horses.

Table 5 - ***Average hay intake and total estimated fructan intake, of geldings on either a high (18% DM) NSC hay or moderate (12% DM) NSC hay during each period.***

	Period 1		Period 2	
	High NSC Hay	Moderate NSC Hay	High NSC Hay	Moderate NSC Hay
Hay (kg/horse/day)	7.7 ± 0.9 [a]	8.0 ± 0.8 [ab]	7.8 ± 1.0 [ab]	8.6 ± 1.1 [b]
Estimated Fructan (g/kg BW/day	0.75 ± 0.03 [a]	0.49 ± 0.02 [b]	0.82 ± 0.07 [a]	0.50 ± 0.02 [b]

Values are Mean ± SD; BW = Body weight; Estimated fructan = WSC-ESC. Values with different superscripts within a row differ ($p<0.05$).

The definition of hyperinsulinemia in horses depends upon the fasted or fed state and the reference interval used; >20µIU/L after a 6-hour fast (Frank et al., 2010) to >40µIU/L (Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY) in non-fasted, forage-fed horses. A defined fast was not applied prior to blood collection; however, geldings were offered their evening meal 16 hours prior to the morning sampling and subjectively finished the evening meal within 4 hours.

Geldings in the present study were fed each of the two hays for a 28-day period and were not transitioned to either hay. The largest increase in insulin concentrations for the geldings fed the high NSC hay was between day 0 and day 7. The change from the acclimation pasture or washout period hay to the high NSC hay would represent an increase of dietary NSC by 156% and 83%, respectively. An increase in enzymatically digestible carbohydrates should result in

increased absorbed glucose and increase serum insulin. Of interest is the decline in insulin after day 7. Perhaps this decline represents decreased insulin secretion relative to the diet, increased insulin utilization by the tissue or increased clearance of insulin by the liver.

Feeding a measured amount of hay daily was initiated to control for variation in total hay intake. Due to the younger stage of maturity at which the grass was harvested, the high NSC hay was lower in both acid detergent fiber and neutral detergent fiber, meaning that the high NSC hay was both more digestible and less bulky. Geldings would have likely consumed more of the high NSC hay if offered free choice, which may have influenced insulin concentrations in geldings.

Alternate tests such as the frequently sampled intravenous tolerance test, euglycemic hyperinsulinemic clamp, or glycemic/insulinemic response may have revealed variations in insulin dynamics not expressed in the present study. However, the use of dynamic tests was beyond the scope of this study as our objective was to evaluate the effect of high and moderate NSC hay on baseline insulin, glucose, leptin, and triglyceride concentrations in overweight Arabian geldings. The authors recognize the limitations of single daily sampling as there are diurnal fluctuations in blood insulin concentrations in the horse. However, the utilization of single baseline blood sampling mimics practical methods for screening horses for metabolic disease in private practice. Repeating such study with obese insulin resistant horses would yield further information to be applied to the EMS horse.

Dyslipidemia has been associated with IR and obesity in horses (Frank et al., 2006; Garcia and Beech, 1986); therefore, plasma triglyceride concentrations were evaluated in these overweight geldings. Triglyceride concentrations increased during period 1 and decreased during period 2, independent of diet. Although triglyceride concentrations were higher at the

start of the second period than the first period, mean concentrations remained within the reference interval for horses (11-57mg/dl; Clinical Pathology Lab, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA). Geldings both lost weight during period 1 and were fed less DE during period 1 as compared to period 2 (Table 4). When energy intake is not adequate, hormone sensitive lipase is stimulated to mobilize fatty acids from adipose tissue, subsequently the liver can repackage these fatty acids into triglycerides (Frank et al., 2002). The difference in body weight changes and energy intake between periods likely explains the effect of period on serum triglyceride concentrations. An effect of hay on triglyceride concentrations was not detected and geldings did not develop hypertriglyceridemia during the study.

Leptin is produced in the adipose tissue and potentially regulates appetite and energy balance. Leptin is negatively correlated with insulin sensitivity and positively correlated to body condition score (Buff et al., 2002; Frank et al., 2006). High leptin concentrations are associated with increased insulin concentrations (Cartmill et al., 2003), and increased leptin concentrations have been positively correlated with risk of laminitis (Carter et al., 2009b). In the present study, leptin concentrations remained low (<5ng/ml) (Cartmill et al., 2003) throughout the study and did not differ between groups even though geldings were overweight. Findings in the present study are similar to those from Cartmill *et al*. (2003) where a group of obese horses maintained low leptin concentrations throughout the study. Furthermore, geldings in the Cartmill *et al*. (2003) study had lower leptin concentrations than mares in the study.

In the present study, overweight Arabian geldings fed high (18% DM) NSC hay for 28 days had higher insulin concentrations on day 7 as compared to geldings fed moderate (12% DM) NSC hay. An effect of hay on insulin concentrations on days 14, 21, and 28 was not detected. In this study, the ability for a high NSC hay to increase serum insulin did not persist.

Effects of the different hays on blood glucose, leptin, and triglycerides were not detected. In conclusion, cool-season grass hay with high NSC concentrations, when hay is fed to meet daily energy requirements, does not result in hyperinsulinemia in sedentary overweight Arabian geldings.

3.6 - Acknowledgements

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Section 4 - Evaluation of the Fecal Microbiome of Arabian Geldings Fed a High and Moderate Non-Structural Carbohydrate Hay

4.1 - Abstract

Hindgut microbes enable the horse to thrive on a forage-based diet and changes in the equine hindgut microbiome are associated with disease, such as laminitis. Large oral doses of non-structural carbohydrates (NSC) alter the equine hindgut microbiome and may cause laminitis. Cool-season grasses can accumulate high concentrations of NSC; however, the role of cool-season grass NSC on equine hindgut microbes has not been evaluated. Most gut microbes cannot be cultured at present; therefore, culture-independent molecular methods are more appropriate for exploring these microbes. The majority of culture-independent techniques rely on bacterial DNA extraction for evaluation and feces is a complex matrix to extract bacterial DNA from. The objectives of this study were to (1) evaluate the use of commercial DNA extraction kits in extracting microbial DNA from horse feces and (2) to characterize changes in the fecal bacteria diversity and abundance of overweight Arabian geldings fed a high (18%) vs. moderate (12%) NSC hay using denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR).

Fecal samples were obtained from eight Arabian geldings fed high (18%) and moderate (12%) non-structural carbohydrate hays in a cross-over design during two 28-day periods. Fecal bacteria were fingerprinted by amplifying the 16S rRNA gene and separating amplicons using DGGE. Community similarities between samples were assessed by fecal bacterial 16S rRNA sequence banding pattern similarity indices. The abundance of numbers of 16S rRNA copies of fecal bacteria and *Firmicutes* phylum members were determined.

Due to the nature of sample collection, samples were not collected from each horse on every sampling day. The number of DGGE bands within each fecal microbial sample did not differ (p=0.779) between Arabian geldings fed a 18% NSC hay (mean 45.4 bands [range 37-59]) and 12% NSC hay (46.3 bands [34-61]). Similarity indices between fecal bacterial community fingerprints from samples collected within day (AM vs. PM) were not consistently increased as compared to similarities between samples collected on subsequent days; furthermore, similarity indices between day and within horse ranged from 60-88%. Increased similarity between fecal bacterial community fingerprints was seen for DNA extracted using the Zymo Soil Microbe DNA Kit (80.5-87.9%) compared to the MoBio Ultra Clean® Fecal Kit (63.8-79.6%). The abundance of total bacteria (p=0.05) and *Firmicutes* (p=0.02) only slightly increased in the feces of 3 geldings fed 12% NSC hay (mean 8.24 range [8.19-8.30] and 8.06 [8.03-8.11] $\log_{10}$ copies/g feces, respectively) compared to the feces of the same 3 geldings when fed the 18% NSC hay (8.11 [8.06-8.16] and 7.97 [7.97-7.98] $\log_{10}$ copies/g feces, respectively). However, this difference was only detected with one of three primer sets evaluated. An effect of high (18%) vs. moderate (12%) NSC hay on the fecal microbiome in Arabian geldings was evident from real-time PCR data, but not from the DGGE data. Due to limitations of the present study, conclusions regarding the relevance of higher total bacteria and *Firmicutes* in the feces of horses fed the moderate (12%) vs. high (18%) NSC hay is beyond the scope of this study.

4.2 – Introduction

The horse relies on microbes within the hindgut (cecum and large intestine) for provision of calories (through volatile fatty acid production) (Vermorel and MartinRosset, 1997), nutrients (i.e. B-vitamins, vitamin K), immune stimulation, and pathogen exclusion. In horses fed a forage diet, members of the *Firmicutes* phylum dominate (72% total bacterial) the equine hindgut

bacterial community (Daly et al., 2001). Large oral doses of non-structural carbohydrates (NSC), like those found in forage (i.e. 10g fructan per kg body weight), is associated with a rise in the abundance of equine cecal *Firmicutes* phyla members, specifically *Streptococcus* spp., (Milinovich et al., 2008). Fructans are soluble fibers that are rapidly fermented in the hindgut into lactate, which decreases lumen pH (Milinovich et al., 2007). Furthermore, the reduction in lumen pH and increase in *Firmicutes* phylum member abundance in the equine cecum is associated with the onset of laminitis. Therefore, changes in the equine hindgut bacterial community is associated with disease and indirectly linked to dietary carbohydrates. Cool-season grasses can accumulate high concentrations of NSC, particularly levan-type fructan (28% forage DM), and grass consumption has been implicated in the onset of laminitis in horses (Longland and Byrd, 2006; USDA, 2000). Veterinarians recommend feeding forage with less than 10% NSC (dry matter basis) to horses at risk of laminitis (Frank et al., 2010). However, the effect of cool-season grass NSC on hindgut microbes in horses has yet to be fully elucidated.

The prevalence of overweight and obese conditions in adult horses fed mostly forage diets is 54%, collectively (Thatcher et al., 2008). Obesity is associated with hyperinsulinemia and insulin resistance; all factors increase the risk of laminitis in horses (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007). Therefore, overweight horses may be at increased risk of adverse effects of forage carbohydrates on hindgut microbes. Furthermore, an increase in the relative abundance of *Firmicutes* phyla members is linked to obesity in humans (Ley et al., 2006a) and rodents (Ley et al., 2005; Turnbaugh et al., 2008). The relationship between hindgut microbes and body condition has not been evaluated in the horse. Therefore, a better understanding of the gut microbiome of overweight, but otherwise healthy, horses fed cool-

season grass will likely improve the interpretation of hindgut microbiome alterations in diseased horses.

The majority of gut microbes cannot be cultured and molecular methods for evaluating such a complex bacterial population are expanding. The first step in evaluating bacteria in the majority of molecular methods is DNA extraction. Feces is a challenging matrix to extract DNA from as there are bacterial cells, animal cells (i.e. sloughed epithelium), plant cells, and a variety of molecules present (i.e. polysaccharides, polyphenols, bile, etc.). DNA can be extracted from cells and tissue by use of commercial kit methods and non-kit protocols. Commercial DNA extraction kits reduce human exposure to toxic reagents and streamlines the extraction process, which enables a more samples to be processed in each run (Monteiro et al., 1997). Commercial kits have been used to extract equine gut/fecal microbial DNA (Gronvold et al., 2010; Hastie et al., 2008; Willing et al., 2009); however, several authors report the use of non-kit protocols; such as, phenol-chloroform (Daly et al., 2001; Daly et al., 2011) and phenol EDTA (Yamano et al., 2008).

Two challenges associated with DNA extraction include appropriate cell lysis and elimination of molecules that negatively impact down-stream DNA analysis, particularly DNA polymerase inhibitors. The first challenge is with the first step of DNA extraction, cell lysis. Cell lysis should be aggressive enough to break apart the cell; however, cell lysis that is too aggressive will result in DNA damage (i.e. DNA fragmentation/sheering). Bacterial cell lysis methods in commercial DNA extraction kits include physical or chemical/enzymatic methods. Ariefdjohan *et al.* (2010) reported that aggressive physical lysis methods (bead beating with vigorous shaking) resulted in higher fecal DNA yield than chemical/enzymatic lysis or bead beating without vigorous shaking. Furthermore, DNA quality was negatively correlated with the

quantity of feces used for DNA extraction (highest quality with 0.1 and 0.25g feces; lowest with 1.0 and 2.0 g feces). Furthermore, feces contain both Gram-positive and Gram-negative bacteria and uniform cell lysis of Gram-positive and negative cells is a challenge because of structural differences in the cell wall (Todar, 2008). Gram-positive bacteria cell walls are composed of a thick peptidoglycan layer; Gram-negative bacteria have a thin peptidoglycan layer, which is protected by lipopolysaccharide (LPS) layer. Different cell lysis methods between commercial extraction kits may result in some degree of difference between which cells are lysed and thus influence results of DNA-based bacterial community evaluation. Therefore, molecular analyses of bacterial DNA should be conducted on samples extracted using the same method across samples.

The second challenge with extraction of microbial DNA from feces is elimination of organic molecules such as protein, polysaccharides (Monteiro et al., 1997), bile (Abu Al-Soud et al., 2005), and polyphenolic molecules (i.e. humic acids derived from decomposition of plant material, such as lignin and tannin) (Kreader, 1996; Ogram et al., 1987; Tsai and Olson, 1992). Inadequate elimination of these organic molecules can negatively impact the function of DNA polymerase during down-stream PCR reactions (Ariefdjohan et al., 2010). Monteiro *et al.* (1997) suggested that the polysaccharides remaining after commercial DNA extraction, using the QIAamp tissue method (Qiagen, Valencia, CA), are negatively charged since they remain with DNA after extraction. Fiber polysaccharides are present in horse feces since only 37% (Karlsson et al., 2000) to 64% (Staniar et al., 2010) of the fiber fraction of a horse's diet is digestible (apparent digestibility). However, cellulose and starch are neutral polysaccharides (Whistler and BeMiller, 1997) and the degree of fecal cellulose carry-over during DNA extraction is unknown to the author's present knowledge. Pectin, a negatively charged polysaccharide (Gruber, 1999) is

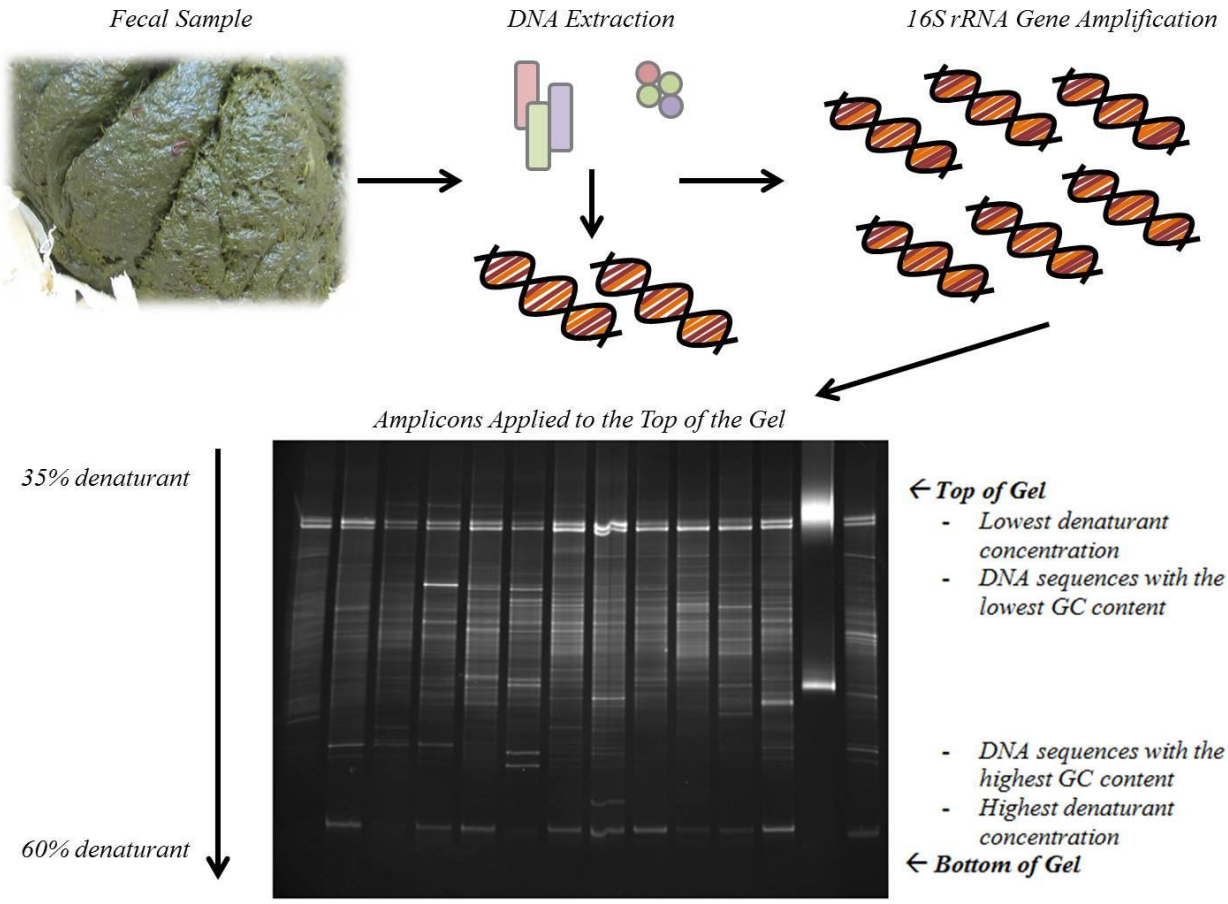
a soluble fiber found in forages and is rapidly fermented by equine hindgut bacteria (NRC, 2007). However, the apparent digestibility of pectin, or presence/abundance of pectin in horse feces, has not been reported in the horse. Lipopolysaccharides, a component of Gram-negative outer membrane, may also contribute to the total polysaccharides found in feces (Chan and Goodwin, 1995).

The quantity of extracted DNA can be evaluated by spectrophotometry or quantification of fluorescence using DNA-binding dyes (i.e. Hoechst, ethidium bromide). Spectrophotometry is a rapid (few minutes) method for evaluating extracted DNA; output includes DNA concentration (ng/µL), 260/280 ratio, and 260/230 ratio. DNA, protein, and other organic molecules absorb light at 260nm, 280nm, and 230nm, respectively; therefore, the 260/280 and 260/230 ratios of DNA extracts can be used to estimate DNA purity. A 260/280 ratio of 1.8 represents high quality/pure DNA; a ratio $\geq$ 2 is suggestive of a high quantity of RNA (Doshi et al., 2009; Sambrook and Russell, 2003). A 260/280 ratio <1.8 indicates a high concentration of protein is present, which may prevent accurate DNA quantification. A 260/230 ratio >1 means that there is more DNA present than organic compounds (i.e. carbohydrates) and a ratio of 2.0-2.2 is considered ideal (NanoDrop Technologies, 2007). A low 260/230 ratio is indicative of a high concentration of organic compounds is present in the sample (Doshi et al., 2009; NanoDrop Technologies, 2007) and these organic compounds can alter down-stream DNA polymerase function.

The two molecular techniques used to evaluate fecal bacteria in the present study are denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR). Denaturing gradient gel electrophoresis (Endo et al., 2009; Milinovich et al., 2008; Muyzer, 1999; Muyzer et al., 1993) is a relatively low-cost PCR-based molecular fingerprinting technique

for evaluation of the genetic diversity of a bacterial population. In DGGE, microbial DNA is extracted from a sample and a 200-700bp portion of the 16SrRNA gene is amplified with primers targeting the 16S rRNA gene of all bacteria (Muyzer et al., 1993). The forward primer has an additional 20-50bp guanine and cytosine (GC) sequence that functions to prevent complete DNA denaturation on the DGGE gel. The 16S rRNA amplicons are then applied to a polyacrylamide gel containing a vertical denaturing gradient and individual 16S rRNA sequences are separated along the denaturing gradient based on G+C content (Figure 5). The polyacrylamide gel in DGGE is run under an ion gradient such that the top of the gel (where samples are loaded) is under negative charge and the bottom of the gel is under positive charge. Gels are then stained and imaged (Figure 5). Bands on gels are digitized and the banding pattern similarities of each microbiome are compared using computer software. Individual bands may be isolated and sequenced to identify the microbial DNA present in each band.

Figure 5 - ***Overview of the steps involved in DGGE from fecal collection to gel.***



Banding patterns representing the 16S rRNA amplicons prepared from each sample. Each lane represents a sample and each band represents bacterial 16S rRNA gene amplicons with similar GC content.

The resultant bacterial community fingerprints (16S rRNA gene banding patterns) may then be compared between samples. Denaturing Gradient Gel Electrophoresis has been used to characterize bacterial community changes in the gut of dogs (Suchodolski et al., 2004; Vanhoutte et al., 2005), mice (McCracken et al., 2001), sheep (Liu et al., 2012), and humans (Vanhoutte et al., 2004; Zwielehner et al., 2011). The analysis of DGGE banding patterns is not reliant on the database of known microbes; therefore, DGGE allows for a quick overall comparison of multiple bacterial communities when a high number of unknown or unclassified bacteria are present. The intensity of each DGGE band is suggestive of increased abundance of DNA sequences with similar GC content. However, band intensity does not accurately correlate with bacterial abundance because of potential co-migration and the multiple copy nature of the bacterial 16S rRNA gene.

Real-time PCR is a quantitative method useful for evaluating the abundance of individual bacteria or members of targeted bacterial groups (Smith and Osborn, 2009) and has been used to evaluate the abundance of bacteria in the equine hindgut and feces (Hastie et al., 2008). Real-time quantitative PCR relies on detection of fluorescent signals during amplification in "real-time" and thus can capture the initial gene copy number (Heid et al., 1996). The number of thermocycles needed before gene amplification is detected during a real-time PCR reaction (Ct; threshold cycle) is negatively correlated with starting gene copy number. The major limitation of real-time PCR is that assay sensitivity is limited by primer homology to the target gene sequence (i.e. 16S rRNA gene) in the target bacterial population (i.e. *Firmicutes* phylum). Currently there are no primers designed specifically to evaluate the abundance of the *Firmicutes* phylum in equine gut/fecal samples.

4.3 - Objectives

The broad objective of the present study was to use culture independent methods to evaluate the equine fecal microbiome. Specific objectives were to:

- Determine if fecal preparation and subsequent DNA extraction method would influence fecal bacterial banding patterns by using DGGE.
- Determine the appropriate DNA extraction method that would yield the highest amplification of total bacteria and *Firmicutes* phylum 16S rRNA genes.
- Evaluate the effect of sample collection day and time on the variation in the equine fecal microbiome using DGGE.
- Evaluate variation in the equine fecal microbiome of geldings fed an18% vs. 12% NSC grass hay and the effect of diet change using DGGE.
- Evaluate the utility of previously designed 16S rRNA gene primers for evaluation of the abundance of equine fecal total bacteria and *Firmicutes* phylum members.
- Compare the abundance of total bacteria and *Firmicutes* in the fecal microbiome of geldings fed 18% vs. 12% NSC hay by use of real-time PCR.

My hypotheses were that:

- Fecal preparation and DNA extraction methodology will influence the equine fecal microbiome as evaluated using DGGE.
- The gelding fecal microbiomes would not vary within or between days when geldings were fed the same hay for 3 weeks.
- The gelding fecal microbiomes would change during the first 4 days of switching from a 10% NSC grass hay (wash out) to an 18% NSC grass hay.

- The fecal microbiome of overweight Arabian geldings fed the high (18%) NSC hay would have a unique fingerprint, increased number of DGGE bands, and a higher abundance of total bacteria and *Firmicutes* as compared to the fecal microbiome of moderate (12%) NSC hay-fed Arabian geldings.

4.4 – Materials and Methods

4.4.1 - Animals and Sample Collection

The fecal samples for this study were collected from eight Arab geldings utilized in the previous study (Section 3). These samples were chosen as the geldings were in a controlled environment and fed hays of a known nutrient concentration. To minimize environmental contamination and degradation, samples were collected within 20 seconds after voluntary defecation, placed immediately on ice then stored in a –80ºC freezer within 1hr of collection until analysis. Due to the sampling technique, samples were not collected on each horse on each day (Table 6).

Table 6 - ***Fecal sample collection dates and times.***

Horse ID	**229**	**231**	**232**	**241**	**244**	**248**	**249**	**250**
Age (yr)	17	14	22	11	17	19	9	15
BW (kg)	461	410	450	490	589	489	438	439
BCS (1-9/9)	6	7	6	9	9	6	8	7
Period 1								
Hay	12% NSC	12% NSC	12% NSC	18% NSC	18% NSC	12% NSC	18% NSC	18% NSC
12/15/2008		**AM** + PM		AM	**AM** + PM	**AM** + PM		
12/16/2008		AM + PM			AM + PM			
12/17/2008		AM			AM + PM	AM + PM		
12/18/2008						AM + PM		
12/20/2008			AM		AM			
Period 2								
Hay	18% NSC	18% NSC	18% NSC	12% NSC	12% NSC	18% NSC	12% NSC	
1/19/2009		AM	AM		AM	AM	AM	
1/20/2009			PM		AM	AM	AM	
1/21/2009			AM		AM	AM	AM	
1/22/2009			AM		AM	AM	AM	
2/2/2009			AM					AM
2/15/2009	PM	**PM**	PM		**PM**	**PM**		

AM: 0800; PM = 1700; bolded samples indicate those used for DGGE and qPCR analysis of the fecal microbiome of geldings fed the 12% vs. 18% NSC hay

4.4.2 – Equine Fecal Sample Preparation and DNA Extraction

Fecal sample preparation and DNA extraction methods were compared to determine if fecal preparation and extraction method influenced bacterial DGGE banding patterns; furthermore, the fecal preparation and extraction method that yielded the highest bacterial abundance, as determined using real-time PCR, was chosen for subsequent real-time PCR reactions.

Fecal Sample Preparation

Fecal samples were prepared using three methods: whole, homogenized, and homogenized with pelleting as described below. All fecal samples were thawed for 2 hours at room temperature prior to preparation.

Whole Feces - Bacterial DNA was extracted from 0.25g whole, unaltered feces.

Homogenized Feces - Sterile peptone water was prepared as a 0.1% solution (Bacteriological Peptone, Sigma Aldrich). Ten grams of thawed feces was added to 90ml peptone water in a double lumen filter bag (Fisher 01-002-57) such that the fecal sample was placed on one side of the filter. Filter bags were placed in a stomacher (BagMixer 3500 JumboMix, Interscience Laboratories, Weymouth, MA) and mixed on minimum speed for 6 minutes. During stomaching, the liquid and fine particles migrated across the filter to the opposite lumen. Bacterial DNA was extracted from a combination of 0.15g of a mixture of the liquid and fine particles that migrated across the filter plus 0.15g of the fibrous portion of the feces that did not cross the filter.

Pelleted Feces - The liquid and small particle portion from the homogenate, as described above, was poured into 50ml sterile tubes and then centrifuged at 4°C and 4,000 x g for 10 min. The supernatant was discarded. Bacterial DNA was extracted from 0.25g of the remaining fecal pellet.

Fecal Bacterial DNA Extraction

Three fecal samples, samples 12/15/08 from gelding 241, 2/2/09 from gelding 250, and 2/2/09 from gelding 232, were subjected to three DNA extraction experiments. Sample 12/15/08 from gelding 241 was used to determine the appropriate extraction method for DGGE analysis, methods as described in section 4.4.3. Two commercial MoBio DNA extraction kits (Ultra Clean® Fecal and Power Soil®) were used to extract DNA, per manufacturer's instructions, from feces prepared as described above.

Sample 2/2/09 from gelding 250 was used to determine the appropriate extraction method for real-time PCR analysis. Six commercial DNA extraction kits (MoBio Ultra Clean® Fecal, MoBio Ultra Clean® Plant, MoBio Ultra Clean® Soil, Zymo Research Fecal DNA MiniPrep™, Zymo Research Plant/Seed™, and Zymo Research Soil Microbe DNA MiniPrep™) were used to extract DNA, per manufacturer's instructions, from feces prepared as described above. Real-time PCR reactions were carried out as described in section 4.4.4 using total bacteria and *Firmicutes* 16S rDNA-specific primers (Fierer et al., 2005) as listed in (Table 7).

Sample 2/2/09 from gelding 232 was used to expand the fecal preparation and DNA extraction comparison to re-test two previously evaluated commercial DNA extraction kits (Zymo Soil Microbe DNA MiniPrep™ and MoBio Ultra Clean Fecal®) against the Qiagen QIAamp DNA Stool Mini kit. Additionally, amplification of DNA extracted using the Qiagen QIAamp DNA Stool Mini kit was compared using two real-time PCR master mix kits (BioLine SensiMix SYBR® Kit [QT650] and USB HotStart-IT SYBR Green qPCR Master Mix [75762]). Real-time PCR reactions were carried out as described in section 4.4.4 using total bacterial 16S rDNA (Eub 338 + Eub 518) and *Firmicutes* 16S rDNA-specific (LGC 353 + Eub 518) primers (Fierer et al., 2005; Price et al., 2010) (Table 7). *E.coli* DNA (100ng/µL) was also amplified to

serve as a standard to compare the performance of the two real-time PCR master mix kits (BioLine SensiMix SYBR® Kit and USB HotStart-IT SYBR Green qPCR Master Mix).

Evaluation of Extracted DNA

Extracted DNA was assessed for quality and quantity with spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Coleman Technologies). The 260/280 and 260/230 ratios were documented; however, DNA extracts were not altered or discarded based on this data; DNA was re-extracted from a fecal sample only when DNA concentrations were <10ng/µL. All DNA extracts were standardized to a concentration of 60-70ng/µL. Extracts with DNA concentrations < 60ng/µl were concentrated using a vacuum centrifuge (Eppendorf Vacufuge® Plus); extracts with a DNA concentration >70ng/µL were diluted with ultrapure water to the target concentration. DNA quality was further evaluated by:

1. Amplifying DNA and inspecting DNA bands on a 1.5% agarose gel. Quality of extracted DNA was considered adequate if agarose gel bands were crisp (no smearing/DNA fragmentation), bright (high concentration of DNA), equal band brightness across all samples (even amplification across all samples), and presence of a single band per sample (multiple bands per sample is evidence of non-specific amplification during PCR).
2. Evaluating amplification during real-time PCR. Quality of DNA was considered adequate if amplification was detected before 30 cycles (Ct < 30) and data points fell along the standard curve.

4.4.3 - DGGE

Three evaluations were made using DGGE, evaluation the effect of sampling day and time, diet change, and feeding two different hays on the fecal bacteria communities of Arabian geldings. To evaluate the effect of day and time of day on the fecal bacterial community, fecal samples were collected from two Arabian geldings (244 and 248) at 0800 and 1700 over three

days during the last week of the first feeding period. Fecal DNA extracted with the MoBio Ultra Clean® Fecal kit. To evaluate the effect of diet change, from *ad libitum* 10% NSC hay fed on winter pasture to limit-fed 12% or 18% NSC hay fed in individual stalls on gelding fecal bacterial communities, fecal samples were collected from four Arabian geldings (232, 244, 248 and 249) during the first 4 days of the second feeding period. Fecal DNA extracted with the MoBio Ultra Clean® Fecal kit. Finally, to evaluate the effect of feeding a 12% vs. 18% NSC hay on gelding fecal bacterial communities, fecal samples were collected from three Arabian geldings (231, 244 and 248) on the same days during the last week of each period (12/15/08 and 2/15/09). Fecal DNA extracted with both the MoBio Ultra Clean® Fecal and ZR Soil Microbe DNA MiniPrep™ kits to evaluate the effect of DNA extraction on gelding fecal bacterial community profiles.

DGGE PCR

Denaturing gradient gel electrophoresis analysis of equine fecal banding patterns was initially performed using gelding fecal DNA extracted using the MoBio Ultra Clean® Fecal kit and then later repeated using the DNA extracted with the ZR Soil Microbe DNA MiniPrep™ kits due to poor DNA amplification of the Ultra Clean® Fecal kit DNA during real-time PCR analysis (Table 9). A 566bp fragment of the bacterial 16S rRNA gene was amplified using universal primers 341-F and 907-R (Table 4) with a 40nt GC clamp added to the 5' end of the forward primer (Muyzer et al., 2004; Price et al., 2010). Each PCR reaction contained 25µL HotStart-IT® PCR Master Mix 2x (USB 71156, Cleveland, OH), 0.5mM of each primer, and 100ng DNA in a 50µL total volume. The PCR protocol consisted of denaturation at 94°C for 10 min followed by 19 cycles of 94°C for 1 min, annealing at 64°C for 1 min with temperature decreasing 1°C every other cycle, and elongation at 72°C for 3 minutes followed by 9 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 3

min (Lopez-Velasco et al., 2010; Muyzer et al., 2004). The DNA concentration of the PCR products was not quantified by spectroscopy (i.e. NanoDrop), but relative quantity and quality was assessed on a 1.5% agarose gel (Fisher-Scientific, Atlanta, GA), stained with 0.2% ethidium bromide (Fisher BP102), and visualized under UV light.

DGGE Conditions

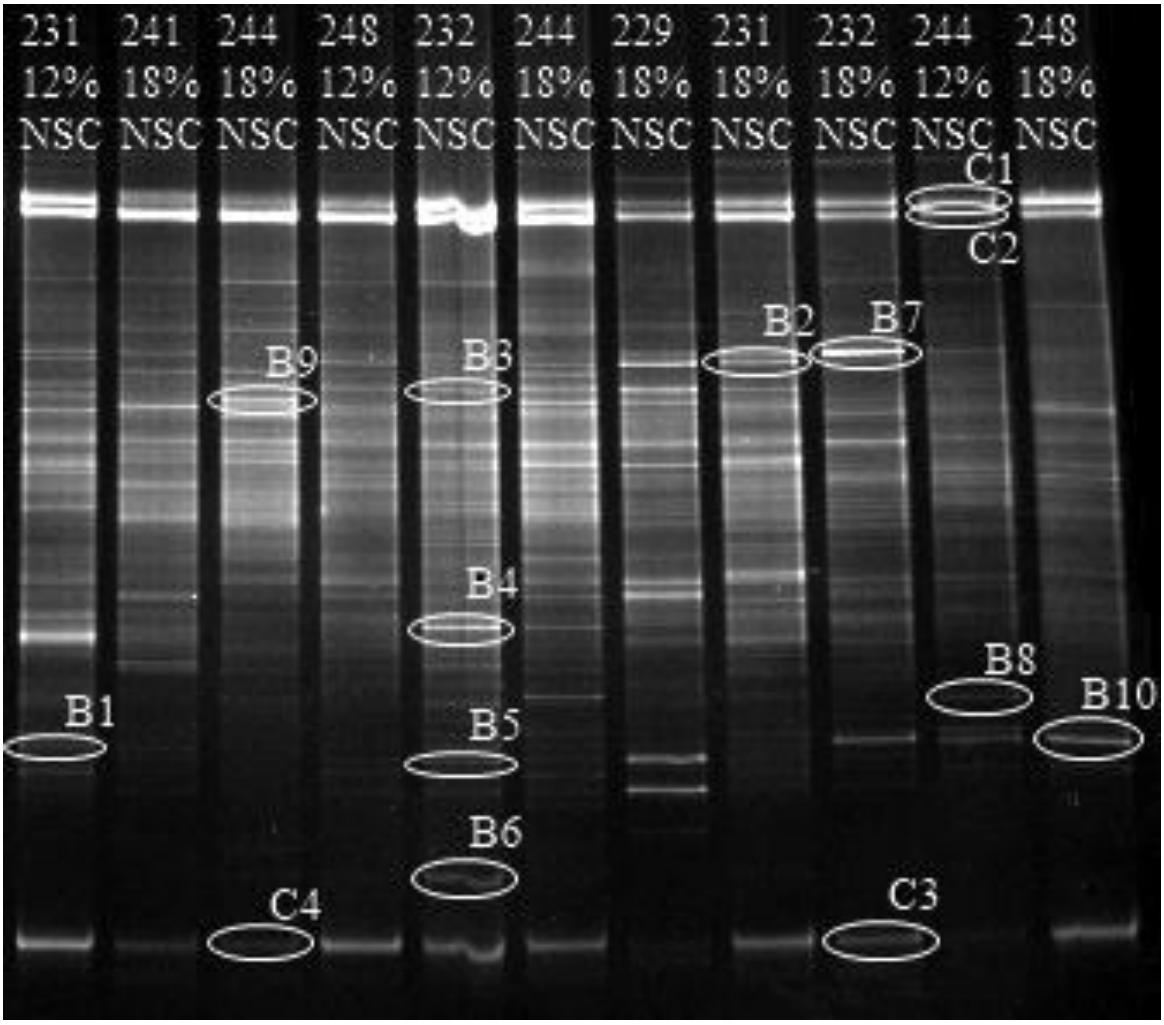
All DGGE analyses were performed using the DCode™ Universal Detection System (Bio-Rad, Hercules, CA). Forty-three microliters of the 16S rRNA PCR amplicons from each amplified DNA sample was applied to an 8% polyacrylamide gel with a 35-60% urea and formamide denaturant gradient as previously described (Lopez-Velasco et al., 2010). The 35% and 60% denaturing gradient solutions are cast together such that the top of the gel (where the samples are loaded) has the lowest concentration of denaturant (35%) and the bottom of the gel has the highest denaturant concentration (60%) (Figure 5).

Electrophoresis was performed at 60°C in TAE buffer (1X Tris-acetate disocium EDTA, Fisher-Scientific) at 85V for 16.5 hours. Gels were stained with ethidium bromide for 35 minutes, and then washed twice in water for 20 minutes. Gels were imaged with a Molecular Imager® GelDoc™ XR (Bio-Rad). Gels were analyzed using FPQuest V.5 software (Bio-Rad Laboratories, Inc., Hercules, CA). The DGGE banding patterns between samples were analyzed using the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) and clustered based on banding pattern similarity.

DGGE Band Cloning

Selected DGGE bands (Figure 6) were excised for nucleic acid identification.

Figure 6 - ***DGGE bands, from Arabian gelding fecal bacteria, isolated for DNA sequence identification.***



The DGGE gel shows DNA bands amplified from DNA samples extracted using the MoBio Ultra Clean® Fecal Kit.

Columns are identified by horse ID and hay type. Isolated bands are identified by letter.

Bands chosen included those conserved across all samples (C1-4) and those unique between hay groups (B1-10). Band isolates are listed in Table 13.

Four bands (C1-4) were selected to represent bands present in all samples (conserved bands). Of the conserved bands, the first two (C1 and 2) were the two prominent bands at the top of the gel, collected from the same sample (fecal sample collected 2/15/09 from horse 244 fed the 12% NSC hay). Bands C4 and C3 represented the same band, but were collected from two different samples (fecal sample collected 12/15/08 from horse 244 fed the 18% NSC hay and fecal sample collected 2/15/09 from horse 232 fed the 18% NSC hay). Ten bands (B1-B10) were selected based on 1) presence in feces of a horse only when fed the 12% or 18% NSC hay (but not when fed both diets) and 2) occurrence as a bright band only in a few samples and faint to absence in other samples. Band 1 and 10 were selected as representatives of the same band across two different samples (fecal sample collected 12/15/09 from horse 231 fed the 12% hay and fecal sample collected on 2/15/09 from horse 248 fed the 18% NSC hay). Band B2 was present in the feces of horse 231 when fed both the 12% and 18% NSC hay and was also present in at least three other samples (very faint in 2 of 5 samples where the band was visualized). Bands B3-B6 were present only when horse 232 was consuming the 12% NSC hay, but not the 18% NSC hay. Band B7 was a bright band present in the feces of horse 232 when fed the 18% hay, but was faint when horse 232 was fed the 12% NSC hay. Bands B8 and B9 were present only on specific days (12/15/08 vs. 12/20/08) during the first feeding period when horse 244 was consuming the 18% NSC hay.

Excised bands were incubated overnight in 100μL sterile nanopure water at 4°C. The following day, diffused DNA was amplified using the same primers as for DGGE-PCR amplification as described above. However, the forward primer did not include the GC clamp (Table 7). The PCR protocol consisted of a single denaturation cycle at 94°C for 10 min, 30 cycles of denaturation at 94°C for 30 sec then annealing at 57°C for 30 sec then extension at

72°C for 1 min, and a single extension cycle at 72°C for 10 min. The 16S rRNA amplicons (15µL) were visualized as individual bands on 1.5% agarose gel. Each band, representing amplified 16S rRNA gene sequences in the selected DGGE bands, was excised from the agarose gel and DNA was purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research #D4001) per manufacturer's recommendations.

The One Shot® Mach1™ Chemically Competent *E.coli* Kit (Invitrogen #K4530) was used to clone each DNA sequence isolated from selected DGGE bands (Appendix M). DNA from transformed *E.coli* cells was extracted using the Gentra PureGene® Kit per manufacturer's directions (Appendix J). Extracted DNA was sequenced at the Virginia Bioinformatics Institute (Blacksburg, VA). A total of 170 cloned sequences were assigned to phylogenetic groups using the RDP *Classifier* (http://rdp.cme.msu.edu/classifier/classifier.jsp), using a Bayesian rRNA classifying algorithm, with a cut-off threshold of 80% (Wang et al., 2007).

4.4.4 - Real-Time PCR

Standards

Three cultures were used as positive controls (Appendix H) to generate standard curves for determining the abundance of total bacteria and *Firmicutes* in gelding feces using quantitative real-time PCR. DNA from pure standard cultures was extracted using the Puregene® DNA purification kit (Appendix J). Extracted DNA was diluted to achieve a series of six ten-fold dilutions to achieve 100, 10, 1, 0.1, 0.01, and 0.001ng/µL concentrations. Standard curves were considered adequate if E = 90-105%, $R^2 > 0.98$ where

- E represents amplification efficiency. For each double stranded DNA (dsDNA), there should be a 2-fold increase in ds DNA per PCR cycle, which correlates with a standard curve slope of -3.32

- R^2 represents how well the data points representing the standard dilutions fit the standard curve.

PCR Reaction and Protocol

Sixty-five nanograms (range 60-70) of template DNA was amplified in a reaction containing 12.5µL HotStart-IT® SYBR® Green qPCR Master Mix 2X (USB 75770 Cleveland, OH) containing 5mM $MgCl_2$, 0.4mM nucleotides, and 10nM fluorescein in addition to 1.3µL each of 16S rDNA forward and reverse primers (10µM; Table 7), 2.5µL 10% DMSO (Fisher-Scientific), additional 1µL 25mg/ml $MgCl_2$, 0.5µL ROX passive reference dye, and 4.9 µL nanopure nuclease free water (Amresco E476) in a 25µL reaction. Primers to determine total bacteria and *Firmicutes* abundance were used from previously published work (Table 7).

Table 7 - *Primers used in Section 4*

Primer	**Sequence (5' → 3')**	**Tm (°C)**	**Annealing Temp. (°C)**	**Target Group**	**Standard**	**Reference**
341 F-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG	84.9	64.0	Universal - DGGE	NA	(Lopez-Velasco et al., 2010; Muyzer et al., 2004)
907R	CCG TCA ATT CCT TTR AGT TT	49.6				
Eub 338F	ACT CCT ACG GGA GGC AGC AG	61.3	53.0	Universal	*E.coli*	(Fierer et al., 2005; Price et al., 2010)
Eub 518R	ATT ACC GCG GCT GCT GG (reverse primer for 353F)	58.7	53.0/ 60.0	Universal/ *Firmicutes*	*E.coli/ Exiguo* spp.	
LGC 353F	GCA GTA GGG AAT CTT CCG	52.5	60.0	*Firmicutes*	*Exiguobacterium* spp.	(Fierer et al., 2005; Meier et al., 1999; Price et al., 2010)

Firm 934F	GGA GYA TGT GGT TTA ATT CGA AGC A	56.6	60.0	*Firmicutes*	*Exiguobacterium*	(Guo et al., 2008b; Price et al., 2010)
Firm 1060R	AGC TGA CGA CAA CCA TGC AC	58.0				
Eub 926F	AAA CTC AAA KGA ATT GAC GG	49.9	61.5	Universal	*Exiguobacterium* spp.	(De Gregoris et al., 2011)
Eub 1062R	CTC ACR RCA CGA GCT GAC	55.9				
Firm 928F	TGA AAC TYA AAG GAA TTG ACG	49.9	61.5	*Firmicutes*	*Exiguobacterium* spp.	
Firm 1040R	ACC ATG CAC CAC CTG TC	55.2				

Primers used for DGGE amplicon generation are marked; all others were used for qPCR data.

The PCR protocol consisted of denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 sec, annealing for 30 sec (see annealing temperature in Table 7), and elongation at 72°C for 30 sec. The melt curve consisted of 95°C for 1 min, 55°C for 1 min, 71 cycles of 60.5°C for 30 seconds increasing the temperature with each repeat. The melt curve was evaluated for a single fluorescent peak per PCR reaction; multiple fluorescent peaks indicate non-specific primer amplification (i.e. primer dimer formation). Each reaction was prepared and carried out in biological and technical duplicates as described (Price et al., 2010) using an ABI 7300 (Applied Biosystems, Life Foster City, CA). Relative abundance of the two phyla was calculated using all bacteria copies.

4.4.5 - Statistical Analysis

Banding pattern similarity indices were calculated using Dice's similarity coefficient (McCracken et al., 2001). Dendrograms were constructed based on similarity of banding patterns between samples using the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) (Lopez-Velasco et al., 2010). Each real-time PCR reaction was run in technical replicates (2 plates) and the results between plates were averaged. Real-time data were analyzed using proc GLIMMIX procedure (SAS Inst. Inc., Cary, NC). Hay (18% vs. 12% NSC) affect was assessed by least squares means. Significance was reported as $P \leq 0.05$.

4.5 - Results and Discussion

4.5.1 - Fecal Collection, Preparation and DNA Extraction

In the present study, fecal samples were collected after voluntary defecation. This process can be time-consuming if the researcher wants to minimize the potential for environmental contamination or degradation of the sample between defection and collection. Conversely, collecting fecal samples after voluntary defecation is the least invasive way to sample the gut

microbiome. Timed rectal fecal collection imposes the need for additional handlers of the horse, rectal manipulation of the horse with the potential for rectal tears, and possible safety issues for the sampler.

The MoBio Ultra Clean® Fecal kit provided the highest DNA yield when used to extract DNA from whole feces (Table 8) as evaluated using sample 12/15/08 from gelding 241.

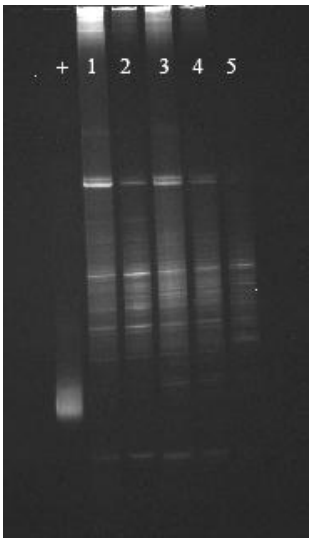
Table 8 – ***Evaluation of fecal preparation methods and two commercial DNA extraction kits***

Kit	**Fecal Preparation**	**DNA (ng/µL elution)**	**DNA (ng/g feces)**	**260/ 280**	**260/ 230**
Power Soil® MoBio 12888 (DNA eluted in 100µL)	Pelleted	4.5	1,800	1.86	0.10
	Homogenized	2.2	880	1.01	0.13
Ultra Clean® Fecal MoBio 12811 (DNA eluted in 50µL)	Pelleted	16.0	3,200	1.19	0.43
	Homogenized	5.5	1,100	1.11	0.44
	Whole	86.1	17,220	1.13	0.46

Fecal sample collected 12/15/08 AM from horse 241

Upon subjective visual evaluation of DGGE analysis (*Figure 7*), fecal preparation and DNA extraction did not appear to influence fecal bacterial community banding patterns.

Figure 7 - DGGE gel representing gelding fecal bacterial community from a single sample (12/15/08 from gelding 241) prepared whole (unaltered) or homogenized +/- pelleting using two different MoBio DNA extraction kits.



Lane ID:

- *+ Positive control*
1. *Homogenized and pelleted feces extracted using the Power Soil Kit*
2. *Homogenized and pelleted feces extracted using the Ultra Clean® Fecal Kit*
3. *Homogenized and pelleted feces extracted using the Power Soil Kit*
4. *Homogenized feces extracted using the Ultra Clean® Fecal Kit*
5. *Whole feces extracted using the Ultra Clean® Fecal Kit*

The MoBio Ultra Clean® Fecal kit and the ZR Soil Microbe DNA MiniKit™ produced the largest DNA yield per g whole feces (Table 9) as evaluated using sample 2/2/09 from gelding 250. Furthermore, DNA from the ZR Soil Microbe DNA MiniKit™ had the highest 260/230 and 260/230 ratios and produced the lowest Ct values (Table 9).

Table 9 – ***Evaluation of two fecal preparation methods and six commercial DNA extraction kits***

DNA Extraction Kit		Fecal Preparation [a]	NanoDrop results				Real-Time Ct Values [b]	
			DNA (ng/µL)	DNA (ng/g feces[a])	260 / 280	260/ 230	Total Bacteria	*Firmicutes*
MoBio	Ultra Clean® Fecal #12811	Whole	**59.2**	11,840	1.13	0.43	27.4	38.3
		Pelleted	22.9	4,580	1.20	0.34	29.0	40.0
	Ultra Clean® Plant #13200	Whole	16.4	3,280	1.29	0.19	22.6	32.8
		Pelleted	**34.1**	6,820	1.15	0.26	28.9	37.7
	Ultra Clean® Soil # 12800	Whole	8.1	1,620	1.16	0.32	18.8	28.8
		Pelleted	**33.3**	6,660	1.14	0.45	28.2	38.2
Zymo Research	ZR Fecal DNA MiniPrep™ D6010	Whole	18.3	3,660	1.28	0.17	18.4	28.4
		Pelleted	6.1	1,220	0.54	0.21	19.5	29.2
	ZR Plant/Seed™ D6022	Whole	21.5	4,300	1.30	0.25	17.7	28.1
		Pelleted	**51.8**	10,360	0.64	0.2	19.0	28.7
	ZR Soil Microbe DNA MiniPrep™ D6001	Whole	**33.1**	6,620	**2.10**	**0.42**	**16.0**	**26.6**
		Pelleted	DNA not detected					

[a] Fecal sample collected 2/2/2009 from horse 250, see the Fecal Preparation in section 4.4.2; [b] PCR reactions were set up using the SensiMix SYBR® (BioLine QT650)

Multiple comparisons were made using sample 2/2/09 from gelding 232. The QIAamp Stool Mini kit yielded higher DNA quantity and quality; the higher 260/230 ratio is suggestive of improved elimination of organic material (Table 10). However, the Ct values for the Qiagen and Zymo kits were similar for whole fecal DNA extracts. The MoBio Ultra Clean® Fecal Kit produced the highest Ct values between the three kits. The mean Ct values were higher when using the BioLine SensiMix SYBR® Kit than when the USB® HotStart-IT® SYBR® Green qPCR Master Mix was used (Table 10). Similarly, the Ct values for the 100ng *E.coli* standard were higher (24.4) for the BioLine SensiMix SYBR® Kit reaction than for the HotStart-IT® SYBR® Green qPCR Master Mix (2X) (16.0).

Table 10 - *Evaluation of three commercial DNA extraction kits*

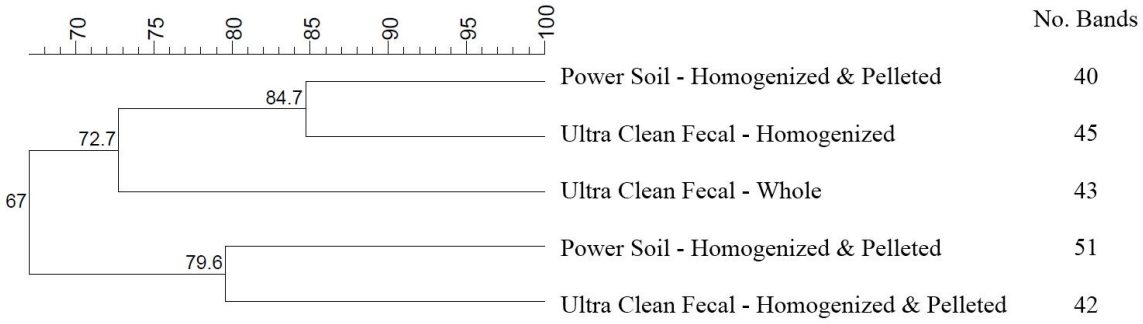
DNA Extraction Kit	Fecal Preparation	NanoDrop results[a]				Real-Time Ct Values			
		DNA (ng/µL)	DNA (ng/g feces)	260 / 280	260/ 230	Total Bacteria		*Firmicutes*	
						Bio-Line	USB	Bio-Line	USB
QIAamp DNA Stool Mini Kit Qiagen 51504	Whole	113.7	45,480	1.82	1.45	20.5	12.7	30.4	17.8
	Pelleted	289.4	115,760	1.77	1.37	22.0	13.2	31.2	17.4
ZR Soil Microbe DNA MiniPrep™ Zymo Research D6001	Whole	33.1	6,620	2.1	0.42	--	11.6	--	17.4
UltraClean® Fecal MoBio 12811	Whole	59.2	11,840	1.13	0.43	--	18.44	--	24.7

[a] *Fecal sample collected 2/2/2009 from horse 232, see the Fecal Preparation in section 4.4.2; DNA was eluted in 100µL (Qiagen) or 50µL (Zymo and MoBio) of buffer (provided with each kit).*

Homogenized and pelleted fecal preparations were used for all subsequent DNA extraction to 1) dislodge bacteria adherent to fiber particles, 2) evenly distribute the bacteria throughout the sample, and 3) to reduce the amount of gross organic debris in the sample. Both the MoBio Ultra Clean® Fecal and Zymo Soil Microbe DNAMiniPrep™ kits were used to extract DNA for subsequent DGGE analysis.

Upon later evaluation of the DGGE banding patterns (Figure 8), by use of objective analysis using dice similarity coefficients, fecal preparation and DNA extraction methods did effect the analysis of the fecal bacterial community from sample 12/15/08 AM from horse 241. The number of bands (representing genetic richness) was numerically higher for the homogenized and pelleted feces extracted using the Power Soil kit (Figure 8).

Figure 8 - ***Dendrogram (UPGMA) generated from DGGE banding patterns of DNA extracted from a single gelding's feces, comparison of fecal preparation and DNA extraction***



Fecal sample collected 12/15/08 AM from horse 241

The DNA yield post-extraction presented here was highest for the Qiagen QIAamp DNA Stool Mini kit and the DNA yield using this kit was comparable to previous reports of DNA extraction from dairy cow feces (25,000ng DNA/g feces) as reported by Yu and Morrison

(2004). Despite the high concentration of DNA recovered using the Qiagen QIAamp DNA Stool Mini kit in the present study, bacterial abundance, as evaluated by use of real-time Ct values did not differ between this kit and the Zymo Research Soil Microbe DNA MiniPrep™ kit. Therefore, the Zymo Soil Microbe DNA Kit was chosen to extract DNA for real-time PCR analysis due to low Ct values and ease of use.

Neither the presence of polysaccharides with DNA post fecal DNA extraction nor the impact that this molecule has on down-stream equine fecal microbial DNA evaluation has been evaluated in the horse. Despite the use of commercial inhibitor removal methods (i.e. ZR Soil Microbe DNA MiniPrep™ IV-HRC column [Appendix K]), the 260/280 and 260/230 ratios for extracted DNA were low in the present study. However, DNA polymerase appeared to be minimally affected as PCR reactions resulted in adequate quality DNA as determined by visualization on agarose gel or amplification during real-time PCR (Table 8, Table 9 and Table 10). Improved removal of proteins, polysaccharides, polyphenols, and potentially other compounds not yet known to impact DNA amplification during DNA extraction may augment PCR DNA amplification.

The USB® HotStart-IT® SYBR® Green qPCR Master Mix was used in subsequent real-time PCR reactions. The higher Ct values associated with amplification of DNA extracted using the MoBio Ultra Clean® Fecal vs. Zymo Research Soil Microbe DNA MiniPrep™ kits could be due to increased presence of humic compounds, or other molecules, that could quench SYBR fluorescence or inhibit DNA polymerase (Zipper et al., 2003). The amplification difference between the two extraction kits could be due to differential cell lysis; however, the cause of the differential amplification between the Ultra Clean® Fecal kit and ZR Soil Microbe DNA kit are beyond the scope of the study; further investigation is warranted. The higher Ct values

associated with using the BioLine SensiMix SYBR® Kit could be due to inferior polymerase stability or activity (i.e. chemical activation vs. non-chemical activation for the USB HotStart-IT SYBR qPCR Master Mix) or differences in kit ingredients as the same reaction volumes and PCR conditions (section 4.4.4.) were used for both kits and the comparison was made on the same plate. The concentration of $MgCl_2$, which positively influences polymerase function, is similar between the kits. The SensiMix SYBR® Kit (BioLine QT650) contains 6mM $MgCl_2$ as compared to the 5mM $MgCl_2$ present in the HotStart-IT® SYBR® Green qPCR Master Mix (USB 75762), which translates to a difference of 0.01molar $MgCl_2$ per 20µL reaction.

4.5.2 - DGGE

DGGE Banding Pattern

A difference in the number of DGGE bands within each gelding fecal microbial sample, representing sequences with differing GC content, was not detected (p=0.779) between the 18% NSC hay (mean 45.4 bands [range 37-59]) and 12% NSC hay (46.3 bands [34-61]).

Similarity indices between fecal banding patterns within day were numerically higher (more similar) for horse 244 (83-87%) than horse 248 (70-84%) (Table 11). Furthermore, similarity indices between fecal banding patterns across the three days were also numerically higher for horse 244 (64 and 88%) than horse 248 (43 and 64%) (Table 11). Furthermore, the similarity index between fecal sample collected from horse 244 between two days (12/15/08 AM and 12/16/08 AM) were more similar (88%) than the samples collected within the first sampling day (AM and PM samples collected 12/15/08) (83%) (Table 11).

Table 11 - *Similarity indices (Dice) of two Arabian geldings' (244 and 248) fecal DGGE banding patterns within and between days.*

A

244	Date	12/15/08	12/16/08	12/17/08
	Time	AM	AM	AM
12/15/08	AM	100		
12/15/08	PM	83		
12/16/08	AM	88	100	
12/16/08	PM	73	87	
12/17/08	AM	64		100
12/17/08	PM	63		86

B

248	Date	12/15/08	12/17/08	12/18/08
	Time	AM	AM	AM
12/15/08	AM	100		
12/15/08	PM	84		
12/17/08	AM	43	100	
12/17/08	PM	57	70	
12/18/08	AM	64		100
12/18/08	PM	57		75

Gelding 244 and 248 were fed the 18 and 12%NSC Hay, respectively, during period 1 for three weeks prior to sample collection.

Cells are color-coded from dark to light orange by 80-90% similarity, 70-80% similarity, 60-70% similarity, 50-60% similarity, and 40-50% similarity.

Similarity indices between day within horse ranged from 60-88% (Table 12), as evaluated from fecal samples during the first week of period 2.

Table 12 - *Similarity indices (Dice) of Arabian geldings' (n=4) fecal DGGE banding patterns between day.*

Horse	232	244	248	249
Diet Change	10% to 18% NSC	10% to 12% NSC	10% to 18% NSC	10% to 12% NSC
Date	1/19/09			
1/20/09	88	84	78	88
1/21/09	86	81	60	82
1/22/09	85	78	65	83

Geldings were switched from the 10% NSC washout hay fed ad libitum to either the 18% NSC hay (limit fed to geldings 232 and 248) or the 12% NSC hay (limit fed to geldings 244 and 249) during period 2 in the morning of 1/19/09.

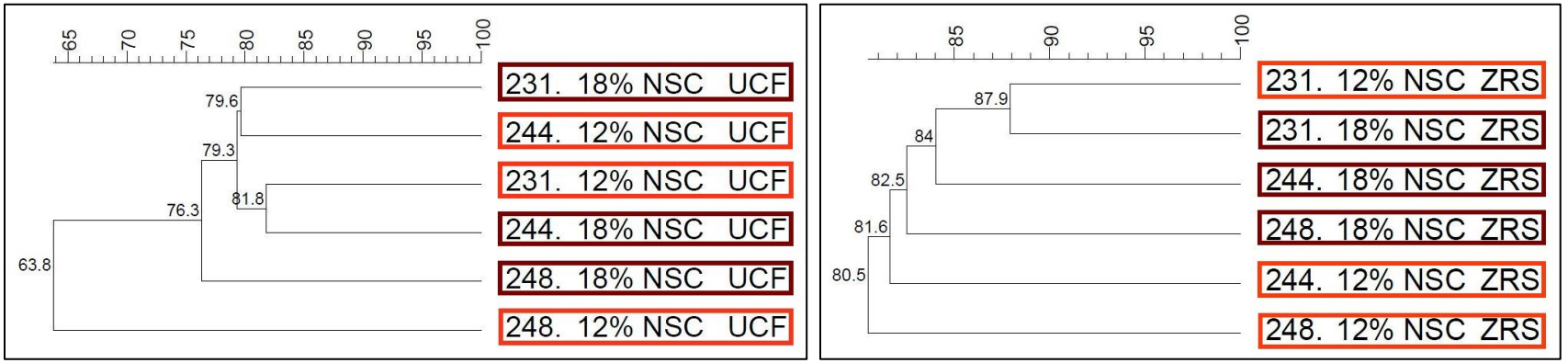
Cells are color-coded from dark to light orange by 80-90% similarity, 70-80% similarity, 60-70% similarity, 50-60% similarity, and 40-50% similarity.

Mean similarity indices between fecal banding patterns of three geldings evaluated when fed the two different hays were numerically higher when DNA was extracted using the Zymo Soil Microbe DNA Kit (80.5-87.9%) than the MoBio Ultra Clean® Fecal Kit (63.8-79.6%)

(Figure 9). Horse 248 shared the lowest fecal banding similarity with the two other horses, particularly when fed the 12% NSC hay. An effect of feeding a high (18%) vs. moderate (12%) NSC hay did not appear to influence the fecal microbiome in Arabian geldings (lack of clustering by hay in Figure 9).

Figure 9 - ***Dendrogram (UPGMA) generated from DGGE banding patterns of Arabian geldings' feces between gelding (n=3) and hay.***

A ***B***



Geldings were consuming the 12% NSC hay (orange) or 18% NSC hay (maroon) for three weeks prior to fecal collection. DNA was extracted using the MoBio Ultra Clean® Fecal kit (UCF; A) or the Zymo Research ZR Soil Microbe DNA MiniPrep™ (ZRS; B)

The fecal DGGE banding patterns in the Arabian geldings were more similar between individuals (80.5-87.9% similarity) (Figure 9) than DGGE banding patterns reported between laboratory hound dogs (28-81%) (Suchodolski et al., 2004) and humans (38-85% in aged [86y/o]; 30-45% in young [24y/o]) (Zwielehner et al., 2009). The broad range in fecal microbial banding patterns across humans likely represents the effect of age, lifestyle, and diet on the human fecal microbiome. Due to the limited number of horses in the present study, an effect of age on the fecal microbiome of Arabian geldings was not evaluated. The hound dogs described by Suchodolski *et al.* (2004) were fed the same canine maintenance diet and housed under the same conditions, but hound body weight, body condition score, and kibble consumption were not reported, which are factors that may influence the gut microbiome. Feeding two different hays did not affect the fecal microbiome of geldings in the present study when environment and diet were controlled. McCracken *et al.* (2001) reported a 67-73% similarity in fecal microbial banding patterns between mice managed under the same environmental and dietary conditions. The geldings in the present study were of the same breed but were not related. Furthermore, the geldings were housed under the same conditions for the study; however, the environment prior to the study (pasture) is very dynamic (nutrient composition changes within and between days). Therefore, there is less variability in fecal bacterial banding patterns between individual horses and between mice than reported between humans. The fecal samples represented in Table 11 were collected during the last week of the first feeding period when horses had been on their respective diets for 3 weeks prior; samples represented in Table 12 were from the first week of the second feeding period when horses were first introduced to the respective hays (12% vs. 18% NSC). An effect of diet change (transition from the 10% NSC wash-out hay to the 12% or 18% NSC hay) may be evident as early as 23-36hr after consumption of the 0800 meal on 1/19/09

based on normal gastrointestinal transit times reported in horses fed forage (Van Weyenberg et al., 2006). Therefore, authors expected to see more variation in the fecal microbiome within horse across consecutive days from day 1/19/09 to 1/22/09, particularly for the horses fed the 18% NSC hay. Of the four horses evaluated during the first week of the second period, both horses 232 and 248 were switched from a 10% NSC hay to a 18% NSC hay on 1/19/2009. Horse 248 presented with a greater change in fecal DGGE banding similarity indices on the first feeding day (60-78% similarity) relative to the other three horses with higher DGGE banding similarity indices (78-88%). Due to the limited sample size of the present study, the reduced similarity between fecal samples for horse 248 cannot be concluded.

In the present study, an effect of a high (18%) vs. moderate (12%) NSC hay on the fecal microbiome of Arabian geldings was not detected (Figure 9). Similarly, Middelbos *et al.* (2007) reported high fecal bacterial DGGE banding pattern similarity (80-88% similarity) between bitches fed diets supplemented with soluble vs. insoluble fiber. However, Huws *et al.* (2010) reported that the rumen microbiome of steers, as determined by DGGE banding patterns, was more similar between individuals when a red clover silage was fed (83% similarity between two individuals) than when a grass silage was fed (68% between two individuals). Although there were few steers in the study, the authors provided evidence of an effect of two different forage-based diets on the rumen microbiome.

Gronvold *et al.* (2010) evaluated the fecal microbiome of 12 horses before and after their respective treatments (penicillin/no penicillin and use of general anesthesia) for a variety of disorders (airway infection, upper airway obstruction, wound, lameness, and arthroscopy) by use of DGGE. Hospitalized adult horses had high variation in individual fecal DGGE banding patterns (21-75% similarity). Six of the 12 horses had different fecal microbial banding patterns

pre and post treatment. The two lowest similarity patterns between fecal bacteria was a horse treated parenterally with penicillin for a wound (21% similarity between pre and post-treatment samples) and one of three horses with an upper airway obstruction treated parenterally with penicillin and fasted general anesthesia (35% similarity between pre and post-treatment samples). Fecal DGGE banding patterns from the two other horses with upper airway obstruction appeared to be less affected (65% and 45%, respectively, pre and post-treatment) by parenteral penicillin treatment. The diet and environment change that horses underwent upon presentation to the hospital may have influenced the fecal microbiomes of horses study by Gronvold *et al.* (2010). Furthermore, although horses were fed ad libitum hay and limited pelleted grain, details regarding the nutrient composition or volumes were not reported.

DGGE Clone Library

Phyla-level identification was obtained in 131 of 170 colonies sequenced from 14 selected bands (Table 14).

Table 13 - ***Bacterial Phyla and Genera-Level Identification of Isolated Arabian Gelding Fecal DGGE Bands***

Phyla	**Genera**	**C1**	**C2**	**C3**	**C4**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**Total**
Horse ID		**244**	**232**	**244**	**244**	**231**	**232**	**231**	**232**	**232**	**232**	**232**	**244**	**244**	**248**	
Hay		**12% NSC**	**18% NSC**	**18% NSC**	**12% NSC**	**12% NSC**	**12% NSC**	**18% NSC**	**12% NSC**	**12% NSC**	**12% NSC**	**18% NSC**	**12% NSC**	**18% NSC**	**18% NSC**	
Actinobacteria	ND			1	1											2
Bacteroidetes	*Prevotella*					1										1
	ND	4	2	8	7	3	3	3	3	6	9	5	7		4	64
Fibrobacteres	*Fibrobacter*												2		1	3
Firmicutes	*Acetivibrio*							1						1		2
	Blautia										1					1
	Roseburia	1														1
	Oscillibacter											1				1
	Phasolarctobacterium											1				1
	ND	2	3				3	9	1			5	1	8		32
Proteobacteria	*Shewanella*	1	2			1	1	2	2	1			2			12
	ND							1					1			2
Spirochaetes	*Treponema*	1		1				1				1	3		2	9

ND = genus not determined.

Members from multiple phyla were isolated in all 14 bands studied (Table 14). The phyla identified were *Bacteroidetes* (n=65 colonies), *Firmicutes* (n=38 colonies), *Proteobacteria* (n=15 colonies), *Spirochaetes* (n=10 colonies), *Fibrobacteres* (n=3 colonies), and *Actinobacteria* (n=2 colonies). Genera-level identification was obtained in only 32 of 170 colonies sequenced (Table 10). The two most commonly identified genera in the present study were *Shewanella* (n=12 colonies) and *Treponema* (n=10 colonies). Other genera identified include *Fibrobacter* (n=3 colonies), *Acetivibrio* (n=2 colonies), and a single colony each of *Blautia, Oscillibacter, Phasolarctobacterium, Prevotella*, and *Roseburia*.

The conserved bands C1 and C2 represented the two bands present in all samples across the top of the gel (Figure 6) and had similar bacterial sequences (Table 13) with the exception of the presence of *Roseburia* spp. and *Treponema* spp. sequences present only in the C1 band. The C3 and C4 conserved bands were collected from two different samples to represent the same band present in all samples at the bottom of the gel (Figure 6). The sequences obtained from these two bands were similar (Table 13) with the exception of the presence of a *Treponema* spp. sequence present only in the C3 band. Bands B1 and B10, representing the same band across two samples, did not contain the same bacterial isolates. Band 1 contained *Roseburia* spp., *Firmicutes*, and *Shewanella* spp. sequences, which were not found in band 10 (Table 13). Furthermore, B10 contained a *Treponema* spp. sequence, which was not isolated from B1. Bands B3-B6 contained *Bacteroidetes* members; however, sequences representing *Acetivibrio* spp., *Blautia* spp., *Oscillibacter* spp., *Phasolarctobacterium* spp., *Shewanella* spp., and *Treponema* spp. were not present in all 4 bands (Table 13).

Selected bands (Figure 6) were sequenced, by way of clone library generation, to identify the bacterial sequences present in each band. The present author expected to find the same

bacteria, in bands that migrated to the same location on the gel (i.e. bands C3 and C4 in Figure 6). Two possible explanations for this are that:

1. Bacterial sequences were present in both bands (i.e. C3 and C4), but were not captured during clone library generation of both bands (i.e. captured only in band C3)
2. These findings represent differences in the microbiome between two fecal samples that cannot be characterized by DGGE alone.

Denaturing gradient gel electrophoresis bands represent bacterial sequences with similar GC content. Species-level identification of bacterial sequences within each selected band is not possible due to the short sequence length of DNA (~540bp) amplified using the Muyzer *et al*. (1993) primers.

DGGE bands represent bacterial sequences with similar GC content (Muyzer et al., 1993; Nakatsu et al., 2000). Therefore, co-migration of different sequences with similar GC content may occur. Vanhoutte *et al*. (2005) sequenced selected canine fecal bacterial 16S rRNA bands directly from the gel without the use of cloning. However, multiple genera were represented along the gel in the present study (across the concentration gradient; top and bottom). In the present study we observed both bacteria with high and low total genome G-C content within the same band (Table 13). This may be due to the limitations of using a single target gene (i.e. 16S rRNA) as the GC content of the target gene may not represent the GC content of the total genome.

Only three phyla, *Firmicutes*, *Bacteroidetes,* and *Spirochaetes*, were identified by clone library generation by Willing *et al*. (2009); however, more total genera were identified from fewer clones in this study. Of the identified DGGE bands, *Treponema* spp. are important hydrogen utilizing, acetogenic bacteria in the gut of termites (Leadbetter et al., 1999). Acetate is

the dominant volatile fatty acid produced in the equine hindgut (Hussein et al., 2004). Perhaps the *Treponema* spp. presence here is suggestive of the role this genera plays in equine hindgut acetogenesis. *Fibrobacter* spp. are important fibrolytic species in ruminants, but there is limited evaluation of the abundance of this fibrolytic genera in the horse (Hastie et al., 2008). *Roseburia* spp. are important intestinal saccharolytic butyrate producers found in human feces and thus may play an important role in maintenance of colonocytes health as butyrate is the primary energy substrate for colonocytes (Duncan et al., 2002b). However, the importance of *Roseburia* spp. in the horse has yet to be determined. *Shewanella* spp. have not been previously described in the horse (Daly et al., 2001; Willing et al., 2009) and are not part of the normal gut microbiome in humans (Nath et al., 2011). *Shewanella* spp. are aquatic bacteria (Beleneva et al., 2009) and likely represent environmental bacteria passing through the equine gastrointestinal tract although the gelding water sources were not evaluated. *Phasolarctobacterium* has not been previously described in the horse (Daly et al., 2001; Willing et al., 2009).

4.5.3 - Real-Time PCR

The abundance of total bacteria ($p=0.05$) and *Firmicutes* ($p=0.02$) were higher in the feces of 3 geldings fed 12% NSC hay (mean 8.24 and 8.06 $\log_{10}$ copies/g feces, respectively) than in the feces of the same 3 geldings when fed the 18% NSC hay (8.11 and 7.97 $\log_{10}$ copies/g feces, respectively) (Table 14). However, this difference was detected only with the DeGregoris *et al.* (2011) primer set.

Table 14 - ***Mean (range) absolute abundance of total bacteria and Firmicutes in the feces of three Arabian geldings (231, 244 and 248) fed a high (18%) and moderate (12%) NSC hay***

	18% NSC	12% NSC	p =
Total bacteria (Fierer et al., 2005) log_{10} copies/g feces	8.23 (8.14-8.36)	8.32 (8.27-8.36)	0.289
Total bacteria (De Gregoris et al., 2011) log_{10} copies/g feces	8.11 (8.06-8.16)	8.24 (8.19-8.30)	**0.047**
Firmicutes (Fierer et al., 2005) log_{10} copies/g feces	7.54 (7.39-7.68)	7.54 (7.50-7.58)	0.962
Firmicutes (Guo et al., 2008b) log_{10} copies/g feces	8.13 (8.07-8.17)	8.21 (8.16-8.23)	0.094
Firmicutes (De Gregoris et al., 2011) log_{10} copies/g feces	7.97 (7.97-7.98)	8.061 (8.03-8.11)	**0.020**

Table 15 - ***Mean (range) relative abundance of total bacteria and Firmicutes in the feces of three Arabian geldings (231, 244 and 248) fed a high (18%) and moderate (12%) NSC hay.***

	18% NSC	12% NSC	P =
Firmicutes (Fierer et al., 2005) % total bacteria	20.9 (17.4-24.7)	16.7 (14.9-18.3)	0.145
Firmicutes (Guo et al., 2008b) % total bacteria	82.8 (62.1-107.0)	78.4 (68.9-90.3)	0.779
Firmicutes (De Gregoris et al., 2011) % total bacteria	72.6 (64.0-81.8)	67.6 (52.9-78.0)	0.612

The abundance of *Firmicutes* was evaluated in the present study because administration of a high NSC diet or oral bolus of NSC, in the form of a high starch concentrate (Daly et al., 2001; Willing et al., 2009) or inulin-type fructans (Milinovich et al., 2006), is associated with increased abundance of *Firmicutes* members, such as *Streptococci* and *Lactobacilli* spp. in the equine hindgut. No studies to date have evaluated the influence of cool-season grass NSC on the equine gut/fecal gut microbiome. The difference in NSC concentrations between the high (18%) vs. moderate (12%) NSC hays in the present study are likely not as high as that between a hay vs. hay and grain diet. Furthermore, a comparison of the total dietary NSC between Willing *et al*. (2009) and the present study may not be made due to lack of information regarding the NSC content of the hay fed in that study. Furthermore, the dietary NSC content was not reported in the Daly *et* al. (2001) study and it can only be assumed that horses fed a ration containing a high starch concentrate would consume more total NSC than those fed a grass hay diet.

The abundance of *Firmicutes*, as shown in the present study using Fierer *et al* (2005) primers, was lower (mean 19% total bacteria) than anticipated based on reports generated by clone-library sequencing from Daly *et al*. (2001) (72% total bacteria). De Gregoris *et al*. (2011) reported that primers described by Fierer *et al*. (2005) only represent only 25% of 16S rRNA gene sequences deposited in the RDP database (http://rdp.cme.msu.edu/), which may explain the apparent low sensitivity of these primers in the present study. Conversely, the abundance of *Firmicutes*, as determined with De Gregoris *et al.* (2011) primers was similar (66% total bacteria) to the work by Daly *et al*. (2001). However, the high relative abundance of *Firmicutes* phylum members, as determined using the Guo *et al*. (2008b) primers, is suggestive of non-specific amplification by amplifying 16S rRNA gene sequences of other phyla members. Primers are named often by the corresponding nucleotide number in *E.coli*, such that primer

Firm934F indicates that this forward primer binds to the 934th nucleotide in the *E.coli* 16S rRNA gene. Therefore, the Firm934F + Firm1060R *Firmicutes* primers described by Guo *et al*. (2008b) would overlap much of the same 16S rRNA gene region as would the Eub926F+Eub1062R total bacteria primers described by De Gregoris *et al.* (2011). This overlap likely accounts for the higher abundance of *Firmicutes* using the Guo *et al*. (2008b) primers because these primers are also targeting non-*Firmicutes* bacteria.

In contrast to the hypothesis of the present study, the abundance of *Firmicutes* was higher in the feces of geldings fed the 12% NSC hay. This may be explained by a higher abundance of fibrolytic members of the *Firmicutes* phyla due to the higher fiber (61% neutral detergent fiber [NDF]) in the 12% NSC hay as compared to the 18% NSC hay (53% NSC DMB). Real-time PCR in the present study was used to explore changes in the abundance of total bacteria and *Firmicutes* members collectively and was not used to explore fecal microbiomes at a deeper taxonomic level. A taxonomically deeper characterization of the *Firmicutes* phylum in equine feces is warranted due to the dominance of this phylum within the equine hindgut and changes previously highlighted in the face of disease (Milinovich et al., 2008).

4.6 - Conclusion

The QIAamp Stool Mini Kit (Qiagen) is a widely used commercial DNA extraction kit and appears to produce superior DNA extracts (Dethlefsen et al., 2008; Dowd et al., 2008; McOrist et al., 2002; Salonen et al., 2010; Zoetendal et al., 2006). However, the present author chose to use the Zymo Research Soil Microbe kit due to similar amplicon generation as with DNA extracted using the QIAamp Stool Mini Kit.

The low similarities between fecal banding patterns of the same sample, as presented in Figure 8, is comparable to the influence of DNA extraction method on the similarity

comparisons between horses fed the 12% vs. 18% NSC hay, as illustrated in Figure 9. However, the same two DNA extraction kits were not compared in these two experiments and the effect of MoBio UltraClean® Fecal and Zymo Research Soil Microbe DNA MiniPrep™ kits, as illustrated in Figure 9, was not compared within a single sample.

DGGE is a relatively rapid method for creating a fingerprint of a complex bacterial population and thus is good for monitoring global changes in these populations. The fecal microbiomes of the geldings, as shown by fecal DGGE banding patterns, shared high similarity. Time and day influenced the DGGE banding pattern representing Arabian gelding fecal microbiomes in the present study. Therefore, the present author recommends collecting fecal samples twice daily (associated with meal time) over several days (i.e. 3-4 days) and either compositing fecal samples within horses prior to analysis or presenting means from fecal analyses within horse. The higher fecal DGGE banding similarity between geldings than between hay groups indicates a greater effect of individual than a high (18%) vs. moderate (12%) NSC grass hay in the present study. Due to the limitations of bacterial identification using DGGE, a second methodology, such as high through-put sequencing (i.e. pyrosequencing) would be a more accurate method for identifying selected bacterial DNA sequences.

Total bacteria and *Firmicutes* abundance differed between the three primer sets used in the present study. The primers described by De Gregoris *et al*. (2011) appeared to have higher specificity than the Guo *et al*. (2008b) primers and higher sensitivity than the Fierer *et al* (2005) primers. The detection of a higher abundance of *Firmicutes* members in geldings fed 12% NSC vs. 18% NSC hay was not expected. These findings warrant further investigation because of the small sample size and need for deeper taxonomic evaluation of gelding fecal microbiomes in the present study.

The effect of cool-season grass levan-type fructans on the equine hindgut microbiome is warranted. Recently, Longland, Dhanoa, and Harris (2012) reported that forage fructan concentration in UK pastures ranged from 8.3% to 29.9g % fructan (DMB), measured by way of high-performance liquid chromatography. This is higher than expected based on calculated fructan concentrations (WSC – ESC = 1-5% DM) in grass hay samples from a US commercial forage lab (Equi-Analytical, 2004b). To compare the effect of inulin vs. levan-type fructan on equine hindgut microbes and test the ability to induce laminitis using levan-type fructans, cecal cannulated horses of equal sex and breed would be divided into three groups, one to receive an oral dose of 7.5g levan fructan/kg BW, the second to receive an oral dose of 7.5g inulin fructan/kg BW, and the third to receive an equal volume of water (control). The fructan dose above was based on consumption the published laminitis induction dose for inulin-fructan (Milinovich et al., 2006; van Eps and Pollitt, 2006) If levan-fructan administration is associated with laminitis, the next step would be to determine the minimum levan-fructan dose needed (0.5, 1.0, vs. 5.0g levan-fructan/kg BW) to induce laminitis. A levan-fructan dose of 0.5g/kg BW is similar to the calculated fructan content from data reported from a U.S. forage lab (3% DM) (2004b); the 1.0 and 5.0g/kg BW fructan doses are those used as a priming (van Eps and Pollitt, 2006) and minimum laminitis induction (Kalck et al., 2009) doses, respectively.

Cecal lumen and fecal samples would then be collected hourly for the first 24 hours and evaluated by use of high-throughput sequencing to compare the cecal microbiome pre and post fructan administration, compare the cecal microbiome post levan vs. inulin-type fructan administration, and if these changes are mirrored in the feces. Furthermore, the effect of cool-season grass fructans on the small intestinal microbiome is warranted due to the link between insulin resistance and fructan administration in ponies (Bailey et al., 2007). Evaluation of the

small intestinal microbiome would be conducted either by use of small intestine cannulated horses (Meyer et al., 1995; Reynolds et al., 1998; Taniguchi et al., 2003) or from post-mortem sampling.

4.7 - Acknowledgement

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Section 5 - Characterization of the Fecal Bacteria Communities of Forage-Fed Horses by Pyrosequencing of 16S rRNA V4 Gene Amplicons

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5.1 - Abstract

The diversity of the equine fecal bacterial community was evaluated using pyrosequencing of 16S rRNA gene amplicons. Fecal samples were obtained from horses fed cool-season grass hay. Fecal bacteria were characterized by amplifying the V4 region of bacterial 16S rRNA gene. Out of 5,898 mean unique sequences, a mean 1,510 operational taxonomic units (OTUs) were identified in the four fecal samples. Equine fecal bacterial richness was higher than reported in humans, but lower than reported in either cattle feces or soil. Bacterial classified sequences were assigned to 16 phyla, of which 10 were present in all samples. The largest number of reads belonged to *Firmicutes* (43.7% of total bacterial sequences), *Verrucomicrobia* (4.1%), *Proteobacteria* (3.8%), and *Bacteroidetes* (3.7%). The less abundant *Actinobacteria*, *Cyanobacteria*, and TM7 phyla presented here have not been previously described in the gut contents or feces of horses. Unclassified sequences represented 38.1% of total bacterial sequences; therefore, the equine fecal microbiome diversity is likely greater than described. This is the first study to characterize the fecal bacterial community in

horses by use of 16S rRNA gene amplicon pyrosequencing, expanding our knowledge of the fecal microbiota of forage-fed horses.

5.2 - Introduction

The horse is a non-ruminant herbivore where the hindgut (cecum and colon) is a fermentative chamber for a complex and dynamic microbial population. Gut microbes serve the host through energy extraction, immune stimulation, pathogen exclusion, and detoxification of toxic compounds. Despite the horse's reliance on these microbes, the scientific literature currently provides limited details on which microbes are present and how these microbes maintain host health.

The horse's forage-based diet is rich in fiber, a molecule indigestible by host enzymes. Hindgut bacteria, especially those with fibrolytic metabolism, enable herbivores to thrive on a high-fiber forage-based diet by slowly fermenting these fibers in the hindgut. The horse's hindgut serves as an ideal anaerobic environment for fiber fermentation. The cecum and colon make up the majority (~70%) of the equine gastrointestinal tract, and 75% of the mean transit time (23-48 hours) is spent in the hindgut (Argenzio, 1975; Van Weyenberg et al., 2006). Ruminant herbivores obtain up to 80% of total daily calories from microbial fermentation with a mean forage retention time of 57 hours (Bergman et al., 1965; Uden et al., 1982). The horse obtains more than 50% of its daily energy requirements from volatile fatty acids that are the microbial products of fiber fermentation (Argenzio et al., 1974; Glinsky et al., 1976; Vermorel and MartinRosset, 1997). In contrast, humans obtain only 10% of total daily calories through fermentation despite having similar mean retention times (Kelsay et al., 1978; Wrick et al., 1983). Species differences could be due to the fact that larger percentages of the gastrointestinal tract of horses and cattle (69% and 76%, respectively) accommodate microbial fermentors in

comparison to humans (17%) (Parra, 1978). Furthermore, the differences in location of microbial fermentation in the horse (hindgut) versus the ruminant (pre-gastric/foregut) may also influence members and functions of these communities. Differences in diet between horses and other species likely also influence the members and function of the microbial communities.

Compared to the rumen microbiota, the equine hindgut microbiota has received little attention; furthermore, few studies have characterized the equine hindgut bacterial community using culture-independent methods (Daly et al., 2001; Daly and Shirazi-Beechey, 2003; Hastie et al., 2008; Yamano et al., 2008). No studies to date have evaluated the fecal bacterial community in adult horses on a controlled forage diet by use of pyrosequencing of 16S rRNA gene amplicons. The objective of this study was to characterize the fecal bacterial community of horses fed grass hay using pyrosequencing of 16S rRNA gene amplicons. We propose that use of high throughput sequencing will provide an evaluation of the equine fecal microbiome, which may be used to increase the understanding of the relationship between the microbes and the host.

5.3 - Materials and Methods

Fecal samples for this study were taken from two adult Arabian geldings during a companion study (Shepherd et al., 2012a). The protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee (#08-217-CVM). Briefly, the geldings were fed orchardgrass hay to meet daily energy requirements for two 28 day periods; fresh fecal samples were obtained immediately after defecation on day 28 of each period. Fresh feces was placed immediately on ice, and stored at -80°C until analysis. The four fecal samples were individually homogenized with 1% (weight in volume) peptone (Sigma-Aldrich Co., St Louis, MO) (10 g feces: 90 ml peptone) in a stomacher for 6 minutes to distribute bacteria throughout the sample (Price et al., 2010). Homogenized samples were centrifuged at 4,000 x g for 10 minutes at 4°C

and pellets were retrieved for microbial DNA extraction. Microbial DNA was extracted from the homogenized fecal pellets using a manual disruption method using the ZR Soil Microbe DNA MiniPrep™ Kit (Zymo Research, Irvine, CA) per manufacturer's instructions (Cuiv et al., 2011; Khafipour et al., 2009). A 270-300 bp nucleotide sequence of the V4 region of the 16S rRNA gene was amplified with primers used by Lopez-Velasco *et al*. (2011) and Jesus *et al*. (2010). Amplicons were generated as described by Lopez-Velasco, *et al.* (2011). Libraries were prepared, enrichments titrated, and pyrosequencing performed using a LR70 sequencing kit and 70x75 PicoTiter Plates™ (2 samples per plate) performed with a Genome Sequencer FLX System (GS-FLX) (Roche, Branford, CT) by the core laboratory facility at the Virginia Bioinformatics Institute (Blacksburg, VA). The reads obtained from GS-FLX were preprocessed to identify sequencing errors and trimmed of linker sequences. Unique sequence taxonomic classification and operational taxonomic unit (OTU) assignment were performed using the Pyrosequencing pipeline of the Ribosomal Database Project (http://pyro.cme.msu.edu/) (Cole, *et al.*, 2009) software tools. Rarefaction indexes were calculated with 3% dissimilarity (http://pyro.cme.msu.edu/). Operational taxonomic unit assignments, estimates of richness (Chao1), and diversity (Shannon index [H']) were calculated at 3% dissimilarity. Evenness was calculated as E=H'/Hmax; Hmax = ln(Chao1) where being S the total number of species in the sample, estimated with Chao1. Relative bacterial phylum abundance was calculated based on the total number of classified reads for each sample using the RDP classifier tool (Table 16). Matches with a RDP confidence estimate below 60% were designated as unclassified bacteria. All sequences have been deposited in the GenBank Sequence Read Archive (accession number SRA039855.1).

5.4 - Results and Discussion

Pyrosequencing of the 16S rRNA gene amplicons was used to characterize the fecal bacteria of healthy adult horses fed a controlled forage diet. Mean length of the pyrosequencing reads and number of reads per sample were 250 bp and 28,458 (range 24,802 – 31,164), respectively. Reads meeting the quality parameters (100% match over 25 bases; minimum of 2 reads) were trimmed. On average 5,898 unique sequences were identified from the 4 fecal samples. The unique sequences classified into 1,510 (range 1414-1778) different bacterial class to order-level OTUs based on 3% dissimilarity, which is greater than reported in the rumen (Hess *et al.*, 2011). The bacterial richness of the horse fecal microbiome presented in this study (Chao 1 = 2,359) is comparable to human feces (2,363) (Larsen et al., 2010) but less than reported for beef cattle feces (5,725) (Shanks et al., 2011), or soil (3,500) (Acosta-Martinez et al., 2008). In contrast the bacterial richess was greater than reported in fecal samples of pigs (114) (Lamendella et al., 2011) or the rumen of cattle (1000) (Hess et al., 2011). Rarefaction curves did not reach an asymptote at 3% dissimilarity (Figure 10); therefore, the richness of equine fecal bacteria is likely greater than described in the present study. Fecal bacterial diversity of the horses in the present study is higher (Shannon Index = 6.7) than that found in swine (3.2) (Lamendella et al., 2011), humans (4.0) (Andersson et al., 2008; Dethlefsen et al., 2008), and cattle (4.9) (Durso et al., 2010) feces. The high fiber nature of the horse's diet and location of the fermentation chamber likely influences this difference in bacterial diversity across species. Bacterial evenness, a measurement of how equally abundant species are in a community, indicates that the species within the horse fecal bacterial community (E = 0.9) are more evenly distributed, and not as dominated by individual taxonomic groups as in humans (E= 0.6) (Dethlefsen et al., 2008).

The majority of sequences classified to the *Bacteria* domain (99%). The remainder sequences (1%) classified to the *Archaea* domain; members of *Archaea* are commonly identified when targeting the 16S rRNA gene V4 region (Yu et al., 2008). The *Methanomicrobia* class, of the *Euryarchaeota* phylum, represented *Archaea* in all samples (mean 47 reads/sample). From all classified bacterial sequences, 10 phyla and 27 genera each represented at least 0.01% of total sequences (Table 17). Sequences from an additional 6 phyla including *Acidobacteria* (0-1 reads/sample), *Deinococcus-Thermus* (0-10 reads/sample), *Chloroflexi* (0-6 reads/sample), *Lentisphaerae* (0-3 reads/sample), *Planctomycetes* (0-1 reads/sample), and SR1 (0-1 reads/sample) were not identified in all samples, suggesting these are rare, possibly transient members of the horse fecal bacterial community. These infrequently occurring phyla, not previously described in the horse, were detected by use of pyrosequencing due to the ability of pyrosequencing to sequence thousands of nucleotide sequences simultaneously. It is unclear if these bacteria are functionally important in the degradation and metabolism of grass forage in horses.

The dominant phyla in each of the 4 samples were *Firmicutes, Proteobacteria, Verrucomicrobia,* and *Bacteroidetes* (Table 16), with the majority of bacterial sequences (43.7%) belonging to the *Firmicutes* phylum. *Firmicutes* and *Bacteroidetes* are the dominant phyla in equine hindgut clone library reports (Daly et al., 2011; Yamano et al., 2008); however, the abundance of *Firmicutes* in the present study is lower than reported by Daly *et al*. (2011) (72%). The *Firmicutes* phylum dominates the bacterial community in pig (55%), human (56%), and beef cattle (70%) feces suggesting an ecological and functional importance of this group within the gut across species (Lamendella et al., 2011; Larsen et al., 2010; Shanks et al., 2011). The abundance of *Bacteroidetes* (3.7%) in the present study is much less than reported in human

(35.4 %) and pig (35%) using high-throughput sequencing technologies (Lamendella et al., 2011; Larsen et al., 2010). The total percentage of *Bacteroides* in this report is also lower than previously reported in horses, however the percentage of *Bacteroides* has been shown to range between 12%-49% of the total number of clones sequenced (Daly et al., 2001; Daly et al., 2011; Daly and Shirazi-Beechey, 2003; Willing et al., 2009; Yamano et al., 2008). These differences may be associated with source of sample, differences in diet and the sensitivity and numbers of clones examined. Daly, *et al*. (2001; 2011) collected colonic samples of euthanized horses that grazed pasture and some received supplemental grain. Yamano, *et al*. (2008) collected fecal samples from horses on bamboo grass pastures. Willing, *et al*. (2009) described a higher abundance of *Bacteroidetes* in horses that were fed an early cut timothy/meadow fescue haylage as compared to horses fed late cut timothy/meadow fescue and concentrate (27%). Unfortunately, a thorough nutrient analysis that documents carbohydrate content is lacking in the previous citations and thus comparisons related to the role of dietary composition are speculative. While it is likely that forage versus concentrate feeding influences the equine gut microbial community to a greater degree than forage alone, the influence of different types of forages on this community has not been determined. In this study, the low relative abundance of *Bacteroidetes* may be in part due to differences in diet, however it is also possible that the primers used are not inclusive of all members of the phyla. Aquatic members of the *Bacteroidetes* phylum have been previously underrepresented by PCR primer-based methodologies (Cottrell and Kirchman, 2000).

Mackie and Wilkins (1988) concluded that the equine bacterial community is dominated by fibrolytic bacteria by use of culture-based techniques. *Fibrobacter* spp. represented 0.75% of total bacteria in the present study, which is similar (1.2%) to cecal contents as reported by

Julliand *et al*. (1999). However, other authors who also quantified *Fibrobacter* spp. by use of oligonucleotide probes reported *Fibrobacter* spp. abundance to be 12% in the cecum and around 4% in the colon (Daly and Shirazi-Beechey, 2003; Lin and Stahl, 1995). When quantified by use of clone library generation, a report of *Fibrobacter* spp. abundance was lower (0.01%) (Daly et al., 2001). *Ruminococcus* spp. and *Eubacterium* spp., additional fibrolytic bacteria described in the horse (Daly et al., 2001; Julliand et al., 1999), represented a sum 0.6% of total bacterial sequences (Table 17). The abundance of *Ruminococcus* spp. in the present study is lower than reported in the hindgut by Daly *et al*. (2001) and Julliand *et al*. (1999) (4.4%). The *Ruminococcus* abundance in equine cecal samples from Julliand *et al*. (1999) are similar to reports in cattle feces (Dowd et al., 2008; Durso et al., 2010). Hydrogen utilizing microbes work with fibrolytic bacteria to produce the volatile fatty acids, like acetate, that the host uses (Robert et al., 2001). *Treponema* spp., a hydrogen utilizing acetogen, represented 1.9% of total fecal bacteria in the present study, which is similar to equine hindgut reports from Daly *et al*. (2001) (3%) and higher than reported in cattle feces (0.93%) (Dowd et al., 2008). Acetogenic *Treponema* spp. compete with methanogens for H+ and the abundance of these two groups is inversely related in the termite gut and human oral cavity (Leadbetter et al., 1999; Lepp et al., 2004). Methane production in the horse is less than that of ruminants (Vermorel, 1997), which may be due to the higher abundance of *Treponema* spp.

Thirteen genera, *Actinobacillus, Asaccharobacter, Denitrobacterium, Acetivibrio, Acidaminococcus, Anaerosporobacter, Blauta, Mogibacterium, Oscillibacter, Papillibacter, Roseburia, Schwartzia,* and *Sporobacter* (Table 17), and three phyla (in addition to the infrequent phyla described above), *Actinobacteria,* TM7, and *Cyanobacteria* (Table 16), that were identified in the present study have not been previously reported in the horse (Daly et al.,

2001; Milinovich et al., 2008; Yamano et al., 2008). The function of the uncultivated bacterial group TM7 (Table 16) in the equine gut is unknown, however this phylum has been identified in the soil and gut of humans, mice, ruminants, and termites (Hugenholtz et al., 2001). Members of the *Cyanobacteria* phylum likely correspond to chlorophyll sequences from the forage diet, however *Cyanobacteria* have been reported in man and mice (Ley et al., 2005), but their role in the equine gut is unknown.

Differences between prior studies and the present study may be due to the culture-independent method employed to study the microbes, biological effect of gastrointestinal tract region, and/or host diet. There is not a gold standard to studying complex microbial populations and the studies reviewed here have represented a variety of techniques that produce some degree of bias due to the preferential cloning of some sequences during 16S rDNA clone library generation (Daly et al., 2001; Willing et al., 2009; Yamano et al., 2008) or use of specific probes for identification of bacterial groups (Daly and Shirazi-Beechey, 2003; Hastie et al., 2008; Lin and Stahl, 1995). Furthermore, PCR primer-based methodologies have underrepresented equine gut bacterial members; such as fibrolytic bacteria (Daly and Shirazi-Beechey, 2003). Gut region appears to influence the abundance of equine gut microbial population (Willing et al., 2009). Feces provide a non-invasive and more humane means to study the gut bacterial community. De Fombelle *et al.* (2003) reported that the number of anaerobic bacteria CFUs differed between the equine hindgut and feces; however, the numbers of cellulolytic bacteria CFUs were similar between the hindgut and feces. Furthermore, Milinovich *et al*. (2007) used nucleic acid hybridization to provide evidence that the relative abundance of targeted groups (i.e. *Streptococcus* spp.) was similar in cecum and fecal samples of healthy horses. However, due to differences described in bacterial community along the equine gut (de Fombelle et al., 2003),

future studies should evaluate gut contents to shed light on the etiology and pathogenesis of chronic diseases that plague horses.

Pyrosequencing provides a rapid and robust description of the equine fecal bacterial community; however, the present study has limitations. These limitations include use of a single region (V4) of the 16S rRNA gene for amplicon generation, generation of short sequence read lengths, inability to achieve a rarefaction asymptote at 3% dissimilarity, and presence of a large number of unclassified sequences. The V4 region of the 16S rRNA gene was targeted for evaluation of equine fecal bacterial communities based on the ability to detect bacterial sequences (Claesson et al., 2009). Kumar *et al.* (2011) reported that the region of 16S rRNA gene amplification does not appear to impact the numbers of rare or abundant taxa detected; however, the relative abundance of several genera was influenced by targeted 16S rRNA gene region amplified. The abundance of *Eubacterium, Prevotella, Streptococcus*, and *Treponema*, as found in human gingiva, varied depending on the 16S rRNA gene amplified (Kumar et al., 2011). Therefore, the abundance of some groups presented here may be biased due to primer selectivity. In this study we did detect groups, TM7, using the V4 region primers that was not detected with the use of V4-V6 primers by Kumar *et al.* (2011). Future studies should use two primer sets spanning different regions of the 16s rRNA genes. The sequence read length was limited by primers utilized; however, the chosen primers have been used previously in bacterial community pyrosequencing studies (Lopez-Velasco et al., 2011; Wang et al., 2007). Furthermore, increasing the specificity by targeting the 16S rRNA gene V4 region helps to overcome the limitations of read length (Nossa et al., 2010). Another source of bias in the present study is DNA extraction technique used; however, Cuiv *et al*. (2011) reported that beading-based extraction is superior for Gram-positive (i.e. *Firmicutes* members) lysis. These

limitations along with the presence of a large proportion of previously uncultivated microbes in the horse feces inhibit complete exposure of the true richness and diversity of the equine fecal bacterial community. In this study, 38% of bacterial sequences were unclassified (Table 16), representing novel bacterial sequences that have not been placed into a recognized taxonomic classification within the RDP database (www.cme.msu.edu) when a 60% similarity cut-off was used. The high abundance of unclassified sequences has been reported in prior human (Eckburg et al., 2005; Gill et al., 2006) and horse (Daly et al., 2001) studies.

This difference between the equine fecal bacterial community and bacterial communities of other environments (i.e. human feces, rumen feces, and soil) may be due to substrate concentration and availability. The horse's diet is markedly different than that of humans (i.e. high fiber and reduced fat, protein, and digestible carbohydrates) and the bacterial environment is different between the hindgut, rumen, and soil. Data presented here provides further insight into the hindgut bacterial community.

5.5 - Acknowledgements

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Table 16 - ***Mean relative abundance of the ten phyla present in the equine fecal samples, in order of abundance.***

Phylum	**%**[1]	
	Mean	**±SD**
Firmicutes	43.73	6.40
Verrucomicrobia	4.11	0.20
Proteobacteria	3.75	1.32
Bacteroidetes	3.65	1.13
Spirochaetes	2.06	1.02
TM7	1.82	0.48
Actinobacteria	1.60	0.53
Fibrobacteres	0.75	0.27
Tenericutes	0.13	0.13
Cyanobacteria	0.12	0.11
Unclassified bacteria	38.14	3.84

[1]*The percentage of sequenced reads was calculated based on the mean total number of reads from the Bacteria domain of the four fecal bacteria libraries using the RDP Classifier tool.*

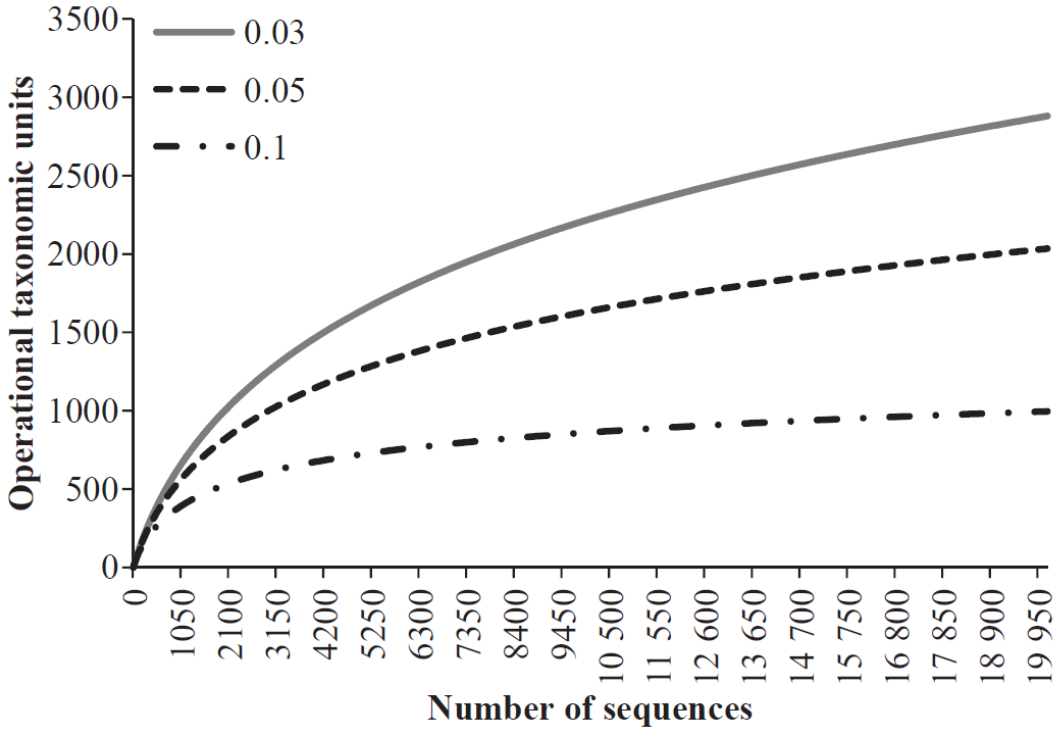
Table 17 - ***Taxonomic identification bacterial genera present at ≥ 0.01% of fecal bacterial sequences, listed in alphabetical order by phylum.***

Phylum	**Genus**	**%[1]**
Actinobacteria	*Asaccharobacter* spp.	0.03
	Denitrobacterium spp.	0.05
Bacteroidetes	*Prevotella* spp.	0.23
Fibrobacteres	*Fibrobacter* spp.	0.75
Firmicutes	*Acetivibrio* spp.	0.19
	Acidaminococcus spp.	0.31
	Anaerosporobacter spp.	0.09
	Blautia spp.	0.92
	Coprococcus spp.	0.23
	Eubacterium spp.	0.09
	Faecalibacterium spp.	0.08
	Lactobacillus spp.	0.36
	Mogibacterium spp.	0.16
	Oscillibacter spp.	0.43
	Papillibacter spp.	0.07
	Pseudobutyrivibrio spp.	0.23
	Roseburia spp.	0.27
	Ruminococcus spp.	0.50
	Schwartzia spp.	0.12

	Sporobacter spp.	0.22
	Streptococcus spp.	0.17
Proteobacteria	*Actinobacillus* spp.	0.03
	Succinivibrio spp.	0.16
Spirochaetes	*Treponema* spp.	1.93
Tenericutes	*Mollicutes* spp.	0.13
TM7	TM7 *Incertae sedis* spp.	1.82
Verrucomicrobia	Subdivision 5 *Incertae sedis* spp.	2.81

[1]*The percentage of sequenced reads was calculated based on the mean total number of reads from the Bacteria domain of the four fecal bacteria libraries using the RDP Classifier tool.*

Figure 10 - ***Rarefaction curves, calculated at 3, 5, and 10% dissimilarity, representing the observed number of OTUs within the 16S rRNA gene-based equine fecal bacterial communities in the four samples combined.***



Section 6 - Diet digestibility, fecal microbial gene expression, and microbial product concentration in overweight and moderate condition mares.

6.1 - Abstract

Obesity is a problem in the equine population. Horses obtain up to 80% of daily energy from products of hindgut microbial fermentation. Obese humans and rodents have a unique gut microbiome as compared to lean individuals. Therefore, overweight and obese horses may have a more efficient hindgut microbiome compared to lean horses. However, the relationship between gut microbes and obesity has not been evaluated in the horse. The objective of this study was to evaluate cool-season grass hay digestibility, plasma and fecal volatile fatty acid (VFA) concentrations, and abundance of total bacteria, *Firmicutes*, *Bacteroidetes*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* in overweight and moderate condition mares.

Ten adult mixed-breed mares (5 overweight; 5 in moderate condition) were limit-fed Orchard Grass hay for a 15d period. During the last 4 days of the study, samples of hay, feces, and plasma were collected for evaluation of hay DM, ADF and NDF digestibility and determination of digestible energy. Digestibility was determined using AOAC methods; digestible energy was measured using bomb calorimetry; plasma and fecal VFA concentrations were determined by use of gas chromatography and mass spectrometry. Bacterial abundance was determined by quantitative real-time PCR using previously designed phyla-specific 16S rRNA gene primers.

A difference in hay digestibility, fecal VFAs, and bacterial abundance was not detected between overweight and moderate condition mares. However, plasma acetate concentrations

were higher (p=0.03) in overweight (1.55mmol/L [1.43-1.65]) vs. moderate condition (1.39mmol/L [1.22-1.47]) mares.

6.2 - Introduction

6.2.1 - Equine Obesity

Obesity and overweight body condition is commonly observed in adult horses, including those fed forage-only diets (Thatcher et al., 2008). Possible causes of obesity include excessive calorie consumption, reduced energy expenditure, or increased digestibility of the diet. Obesity is associated with multiple co-morbidities in the horse, which include reduced reproductive performance (Sessions et al., 2004; Vick et al., 2006), reduced evaporative cooling and reduced athletic performance (Sillence et al., 2006), insulin resistance (Frank et al., 2006; Hoffman et al., 2003; Vick et al., 2007), and increased risk for laminitis (Carter et al., 2009b; Frank et al., 2006; Treiber et al., 2006). Traditional strategies to treat obesity; such as calorie restriction, are typically successful. However, alternative strategies are needed due to poor owner compliance and challenges with calorie restriction for horses allowed to graze pasture *ad libitum*.

6.2.2 - Equine Diet and the Hindgut Microbiome

The equine forage-based diet (pasture or hay) is rich in fiber (i.e. cellulose and hemicellulose), polysaccharides undegradable by host enzymes. Hindgut microbes enable the horse to thrive on a forage-based diet. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* are the most extensively studied fibrolytic bacteria in the ruminant (Denman and McSweeney, 2006; Tajima et al., 2001; Wang et al., 1997). These bacteria have been identified in the hindgut of horses by culture-dependent (de Fombelle et al., 2003; Muhonen et al., 2009) and culture-independent methods (Daly et al., 2001; Daly and Shirazi-Beechey, 2003; Hastie et al., 2008; Lin and Stahl, 1995). Lin and Stahl (1995) evaluated the hindgut microbiome of a pony mare fed alfalfa hay by use of probe hybridization and reported that

Ruminococcus flavefaciens and *Fibrobacter succinogenes* represented 12% of bacteria in the cecum and 4% bacteria in the colon. Hastie *et al.* (2008) reported the abundance of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* 9.71 and 6.70% total bacteria, respectively, in the cecum and 9.31 and 5.53% total bacteria, respectively, in the rectum of slaughtered horses.

6.2.3 - Obesity Microbiome

Obesity is linked to a shift in the composition of the intestinal microbes (gut microbiome) in multiple species (Guo et al., 2008b; Ley et al., 2005; Ley et al., 2006a; Turnbaugh et al., 2009; Turnbaugh et al., 2008). An increase in the relative abundance of the *Firmicutes* phyla and a reduction in the relative abundance of *Bacteroidetes* is evident in the gut of obese humans (Ley et al., 2006a), pigs (Guo et al., 2008b), and rodents (Ley et al., 2005; Turnbaugh et al., 2008). The *Firmicutes* phyla dominates the hindgut and fecal microbiome in horses (44-72% of total bacteria) (Daly et al., 2001; Shepherd et al., 2012b); however, the abundance of *Bacteroidetes* in horses varies from 4 – 49% of total bacteria (Daly et al., 2011; Shepherd et al., 2012a; Willing et al., 2009).

Two of the proposed mechanisms by which obesity is linked to gut microbes are that gut microbes in obese individuals extract more energy from the diet than gut microbes in lean individuals and that gut microbes in obese individuals promote fat storage (Bäckhed et al., 2004). In humans, gut microbes provide an estimated 10% of total daily calories through fermentation (McNeil, 1984). However, hindgut microbes can provide up to 80% daily digestible energy (DE) requirements in the horse (Vermorel and MartinRosset, 1997). The most abundant product of bacterial fermentation of fiber is the volatile fatty acid (VFA) acetate (Argenzio et al., 1974). Acetate is the preferred substrate for fat synthesis in the horse (Suagee et al., 2010); acetate that is not directly used as energy is stored as fat. Therefore, the ability for gut microbes to influence

adiposity is likely much greater in the horse than in humans, yet the relationship between obesity and hindgut microbes has not been evaluated in the horse (Buff et al., 2002; Frank et al., 2006; Thatcher et al., 2008; Vick et al., 2007).

6.2.4 - Methods for Evaluating Forage Digestibility

In vivo diet digestibility is the difference between nutrient consumed and nutrient excreted in the feces. The most commonly reported measure of digestibility is apparent digestibility which is calculated with the following equation: % DM digestibility = 100*((feed DM – fecal DM)/feed DM). Apparent digestibility can be refined by utilizing an estimate for endogenous losses (i.e. secretions, sloughed epithelia tissue) to reflect true digestibility of the feedstuff; however, this is less commonly performed. Apparent digestibility can be measured by use of indigestible dietary markers (Ordakowski et al., 2001; Sales, 2012) or by total fecal collection. There are fewer published reports of the use of indigestible markers, as compared to total collection, for the determination of diet digestibility in horses (Hyslop, 2006; Ordakowski et al., 2001). However, internal markers are useful when diet intake cannot precisely be measured (i.e. evaluating digestibility of pasture forage).

Of the numerous digestibility studies reported in the horse (Crozier et al., 1997; Drogoul et al., 2000; Eckert et al., 2010; Edouard et al., 2008; Hintz et al., 1971; Hussein et al., 2004; Karlsson et al., 2000; LaCasha et al., 1999; Ordakowski-Burk et al., 2006; Staniar et al., 2010; Swyers et al., 2008; Vermorel et al., 1997a), few studies have evaluated individual factors such as breed (Ragnarsson and Jansson, 2011), dentition (Ralston et al., 2001), age (Aiken et al., 1989), and pony vs. horse (Vermorel et al., 1997b). To the author's knowledge, the effect of overweight vs. ideal body condition on adult horse forage digestibility has not been evaluated.

6.3 - Objective

The objective of the present study was to determine the apparent energy, DM, NDF, and ADF digestibilities of grass hay in overweight and moderate condition mares. In addition, fecal and plasma volatile fatty acid concentrations were measured to evaluate primary metabolic outputs of hindgut microbial fiber fermentation. Finally, abundance of members of the *Firmicutes* and *Bacteroidetes* phyla and the abundance of cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in the feces was measured. The author hypothesized that overweight mares would have higher apparent digestibilities, higher fecal and plasma acetate concentrations, higher abundance of fecal *Firmicutes* and cellulolytic bacteria and lower abundance of *Bacteroidetes* than moderate condition mares.

6.4 - Materials and Methods

6.4.1 - Animals and Housing

Ten non-pregnant adult mares (7-23 y/o; 509-606 kg) from the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) teaching herd were allotted to the study. The mares had been managed for at least 3 years as two phenotypically distinct groups based on body condition score (BCS). Five mares were chosen from the moderate body condition group (BCS 4-6) and five from the overweight and obese group (BCS 7-9) respectively on a 9-point scale (Henneke et al., 1983). Average BCS at the start of the study for the obese and moderate condition groups were 7.3/9 and 5.6/9 respectively. The study was designed to detect a 3% difference $\pm$ 1.5% SD in digestibility based on α of 0.05 and 1- β of 0.885.

Mares were housed in individual box stalls (3.6m x 3.6m) with adjacent individual dry paddocks (3.6m x 4.8m) during the 15-day study. The study was divided into two periods: a 10-day acclimation period followed by a 4-day digestibility trial and a final day for morphometric measurements (Table 21). For the first 10 days of the study, stalls were bedded with pine

shavings and mares had access to paddocks 24-hr per day. Stalls were cleaned twice daily (1100 and 2000). For the last five days of the study, stalls were bare except for rubber mats; mares had access to outside paddocks 10-15 minutes once daily during the 1100 hour stall cleaning.

All horses received routine veterinary care including vaccines, deworming, and dental floating prior to the study. The study was conducted during June 2011 in Blacksburg, Virginia (mean ambient temperature 22.1°C). All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC #10-152-CVM).

6.4.2 - Diet

Prior to the study, the mares were housed on cool-season grass (predominantly Tall Fescue [*Festuca arundinacea*]) pastures year around; with supplemental Orchard Grass (*Dactylis glomerata*) hay in the winter months. Mares were housed in individual stalls on day 0-15. Mares were limit fed long-stem Orchard Grass hay (Standlee Hay, Eden, ID) (Table 18) at 2% as fed (AF) of body weight per day divided into two equal feedings at 0800 and 2000. During the first 10 days of the study, the mares were offered a vitamin-mineral supplement (EquiMin® Granular, Appendix C) free choice; this supplement was withdrawn d11-15. Orts were weighed, recorded, and subtracted from the daily amount of hay offered. Total daily dry matter intake was determined for each mare by multiplying total hay intake by hay dry matter (Table 21).

A composite forage sample, representing hay fed to all 10 horses over the 4 sampling days, was submitted for commercial forage laboratory analysis (Trainer Package, Equi-Analytical, Ithaca, NY) of the hay is provided in Table 18; the forage nutrient analysis was determined as described previously in section 3.3.2.

Table 18 - *Nutrient analysis[a] of the Orchard Grass hay fed to mares during the study.*

	Orchard Grass hay [b]
Dry Matter (%AF)	92.4
DE (Mcal/kg DM)	2.02
CP (%DM)	14.5
ADF (%DM)	41.7
NDF (%DM)	62.8
WSC (%DM)	5.8
ESC (%DM)	2.9
Starch (%DM)	0.4
NSC (%DM) [c]	6.2
Ca (%DM)	0.46
P (%DM)	0.45

[a] *Equi-Analytical Laboratories (Ithaca, NY)*

[b] *Values include a single measurement on a composite sample.*

DE = Digestible energy; CP = Crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; ESC = Ethanol soluble carbohydrates (monosaccharides, disaccharides); WSC = Water soluble carbohydrates (monosaccharides, dissacharides, fructan oligo/polysaccharides)

[c] *NSC (calculated) = WSC + Starch*

6.4.3 - Sample Collection

On d0 and d15, mares were weighed on a digital scale (Cambridge Scale Works, Honey Brook, PA); girth circumference, withers height, and body length were measured and BCS

(Henneke et al., 1983) was subjectively scored by a single individual (MLS). Body mass index (BMI) measurements were calculated as previously described (Donaldson et al., 2004; Henneke et al., 1983). Rump fat thickness was measured with a 12mHz tendon probe with the probe placed in the sagittal plane 5cm off of midline at the center of the pelvis (Westervelt et al., 1976); measurements were taken in triplicate and averaged. All measurements were taken before the morning meal; day 0 measurements were taken immediately after transport to the research barn.

Hand grab samples of hay (20g) were obtained at each feeding and stored in brown paper bags until analysis. Total daily feces were collected and weighed on days 11-14. Thrice daily fresh fecal samples were collected; one sample was collected from the rectum at 0800 and two floor samples were collected immediately after defecation within the 1400 and 2000 hour (Table 19). The 20g hay samples and 200g fecal samples were weighed and dried in a 55ºC forced-air oven (Precision Freas Mechanical Convection Ovens Model 645, Pacific Combustion, Torrance, CA) for 96 hours to achieve <10% moisture. Dried hay and fecal samples were then ground 1-mm screen (model 4 Wiley Mill) and subsampled within horse. All digestibility analyses were evaluated in duplicate.

The remaining feces were collected throughout the day into plastic bags kept closed to prevent moisture loss of feces prior to four times daily weighing and disposal. Fecal output was calculated as the summed weight of the four daily fecal weights for each horse.

Blood samples were collected prior to fecal collection and feeding at 0800 into 10ml tubes (BD Vacutainer®, Franklin Lakes, NJ) containing lithium heparin for VFA analysis, in duplicate. Plasma from blood collected into lithium heparin tubes was harvested within 30 minutes of collection after centrifugation (3,000 x g) and stored at -80°C until analysis. Plasma samples were pooled within horse and analysed in duplicate.

Table 19 - ***Fecal samples collected from the mares and the purpose of each collection.***

Collection	**Fecal weight**	**Collection container**	**Storage**	**Purpose**
0800 rectal feces + 1400 & 2000 floor feces	200g	Tin mini loaf pans [a]	Placed in a plastic bag; weighed & placed in drying oven within 2hr of collection	DM, NDF, ADF, and gross energy analysis
0800 rectal feces	50g	50ml tube [b]	Placed immediately on ice; stored at 80°C within 1hr of collection	VFA Analysis
0800 rectal feces	50g	50ml tube [b]	Placed immediately on ice; stored at 80°C within 1hr of collection	Microbial DNA extraction
0800 rectal feces	20g	50ml tube [b] with 25ml RNAlater® [c]	Placed immediately on ice; stored at 80°C within 1hr of collection	Microbial RNA extraction
0800 rectal feces	50g	50ml tube [b]	Placed immediately in liquid nitrogen; stored at 80°C within 1hr of collection	Microbial RNA extraction

[a] *Schneider Paper Products, Inc., Baton Rouge, LA ;* [b]*50ml sterile tube, VWR, Radnor, PA;*

[c] *RNAlater®, Life Technologies, Grand Island, NY*

6.4.4 - Digestibility

Gross energy of ground hay and feces was measured with a bomb calorimeter (Parr 1271A Auto Calorimeter, Moline, IL) using a sample size of 0.15-0.20g (analysis was corrected for sample weight) and jacket temperature at 30°C; 1g benzoic acid was used as the standard and 0.45-0.50g mineral oil was used as the spike. Hay digestible energy (DE) for each horse was calculated using the following, DE (kcal/kg DM) = (gross energy hay [kcal/kg DM] x total daily hay consumption [kg DM]) - (gross energy feces [kcal/kg DM] x total daily fecal production [kg DM]).

Dry matter, ash, ADF, and NDF were determined using AOAC procedures (Appendix U and Appendix V) (AOAC, 2000). Apparent digestibilities of DM, OM, NDF, and ADF were calculated with the following, DM digestibility = (DMI - fecal output)/DMI (Staniar et al., 2010).

6.4.5 - Volatile Fatty Acids

Fecal samples were thawed at 4°C for 4 hours and prepared as described by Otto *et al*. (2003) (Appendix W). Briefly, 2g of thawed feces was mixed with 8ml DI water and 2 drops of concentrated HCl (Fisher-Scientific, Pittsburgh, PA), vortexed for 10 s, and then centrifuged at 25,314 x g for 20 min. The supernatant was filtered through a 0.22µm filter (Millipore Co., Bedford, MA) and stored in 3.7ml (1 fluid dram [DR]) glass vials (#0333922B, Fisher-Scientific, Pittsburgh, PA). Samples were pooled to combine by day within horse and stored at -80°C until VFA analysis. Thawed pooled plasma and fecal supernatant samples were spiked with 100 µl internal standard/volume marker (2.5 mM [1,2-$^{13}C_2$]Sodium acetate, 1 mM [1,2,3-$^{13}C_3$]Propionic acid, 1mM [1,2,3,4-$^{13}C_4$] Sodium butyrate) (Appendix X) then derivatized using a water, acetonitrile, and 2-chloroethanol solution adapted from Kristensen *et al*. (2000) (Appendix X).

Fecal preparations were analyzed for acetate, propionate, and butyrate and plasma was analyzed for acetate by gas chromatography and mass spectrometry (Kristensen, 2000) (Appendix Y).

6.4.6 - Fecal Bacterial Abundance

Bacterial Nucleic Acid Extraction

A commercial kit (ZR Soil Microbe DNA MicroPrep™, Zymo Research, Irvine, CA) was used to extract DNA from 0.25g of homogenized and pelleted feces as described in section 44.4.2. Extracted DNA was quantified as previously described in sections 4.4.2 and 5.3. DNA was standardized to a concentration of 60-70ng/µL.

Two storage methods (RNAlater®-preserved feces and liquid nitrogen preserved feces) and extraction kits (RNeasy® Mini Kit, Qiagen, Ca and Zymo Soil/Fecal RNA Mini Prep, Zymo Research, Irving, CA) with and without a DNase step were evaluated with the goal of optimizing RNA yield and quality from feces (Appendix BB). RNA was quantified with a NanoDrop (Thermo Scientific, Wilmington, DE) and quality was determined using a Bioanalyzer (Virginia Bioinformatics Institute, Blacksburg, Virginia) (Appendix AA, Figure 18). The best combination of quality and quantity was obtained with fecal samples stored in RNAlater® at -80°C, thawed on ice for 2 hour, and isolated with the RNeasy® Mini Kit using the Fungal/Plant protocol with on-column DNase (Appendix BB). RNA was standardized to a concentration of 65-75ng/µL.

Real-Time PCR

The abundance of total bacteria, *Firmicutes* and *Bacteroidetes* phylum members was determined by real-time PCR with SYBR® green primers (Table 20) as described in Section 4.4.5. To evaluate the abundance of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, PCR reactions were set up using TaqMan® primers and probes (Table 20) previously designed by Hastie *et al*. (2008). The *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* primers

used in this study (Table 20) aligned with 92.2% and 93.8% pairwise similarity (Geneious™ alignment) with the *Ruminococcus* spp. (Contig_2985N2) and *Fibrobacter* spp. (Contig_2284N45) sequences, respectively, obtained from the pyrosequencing results (Section 5) (Appendix Z). Each 20µL reaction contained 65ng DNA, 10µL HotStart-IT Probe qPCR Master Mix 2x (75770, USB, Cleveland, OH, USA), 1µL of 20X TaqMan® assay (AB TaqMan® Assay; Table 20), 0.4µL of ROX as passive reference dye (USB 75768), and 7.6µL nanopure water (USB, Cleveland, OH). PCR conditions consisted of one cycle of 2 min at 95°C for activation of HotStart-IT polymerase, followed by 35 cycles of denaturation at 95°C for 15 s, primer annealing and real time detection at 60°C for 30 s, and extension at 72°C for 1 min carried out with an 7300 real time PCR detection system (Applied Biosystem). Standard curves were constructed using 6-fold dilutions of target DNA from pure cultures of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* provided by Dr. Roderick Mackie (University of Illinois, Urbana). Absolute abundance was calculated as $\log_{10}$ copies per g feces.

The abundance of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* 16S rRNA , reactions were carried out as described above with the addition of 0.2µL M-MLV reverse transcriptase (75783, USB, Cleveland, OH) and 0.2µL and RNase inhibitor (75782, USB, Cleveland, OH) with a reduction in the volume of nanopure water to 7.2µL. PCR conditions as described above were preceded by one cycle of 5 min at 50°C for reverse transcription of RNA prior to amplification.

Table 20 - ***Primers used in the present study.***

Primer /Probe	**Sequence (5' → 3')**	**Tm (°C)**	**Target Group**	**Reference**
926F	AAA CTC AAA KGA ATT GAC GG	49.9	Universal	(De Gregoris et al., 2011)
1062R	CTC ACR RCA CGA GCT GAC	55.9	(*Exiguobacterium* spp.)	
Firm928F	TGA AAC TYA AAG GAA TTG ACG	49.9	*Firmicutes*	
Firm1040R	ACC ATG CAC CAC CTG TC	55.2	(*Exiguobacterium* spp.)	
Cfb798F	CRA ACA GGA TTA GAT ACC CT	49.9	*Bacteroidetes*	
Cfb967R	GGT AAG GTT CCT CGC GTA T	54.1	(*Flavobacterium* spp.)	
UniversalF	TCCTACGGGAGGCAGCAGT	60.0	Universal	(Nadkarni et al., 2002)
Universal - Probe	CGTATTACCGCGGCTGCTGGCAC			
UniversalF	GGACTACCAGGGTATCTAATCCTGTT			
*R.flavefaciens*F	GTGTCGTGAGATGTTGGGTTAAGT		*Ruminococcus flavefaciens*	(Hastie et al., 2008)
R.flavefacies - Probe	CCGCAAAGAGCGCAACCCTT			
*R.flavefaciens*R	AGTGCTCTTGCGTAGCAACTAAAG			
*F.succinogenes*F	CGTTCCCGGGCCTTGT		*Fibrobacter succinogenes*	
F.succinogenes - Probe	CACACCGCCCGTCAAGCCATG			
*F.succinogenes*R	CACGACTTAGAGCACTCCCTTCTC			

Tm: melting temperature of a primer, used to determine annealing temperature in a PCR reaction, Tm is positively influenced by G+C content.

6.4.7 - Statistical Analysis

Duplicate digestibility, plasma and fecal VFA, and bacterial abundance analyses were conducted on samples pooled within horse for the collection period. Data was analyzed using SAS (version 9.2, SAS Institute Inc., Cary, NC). A GLIMMIX procedure was used for analysis, with mare within group as the subject, using the following model for analysis:

$Y_{ij} = \mu + G_i + E_{(i)j}$

where Y_{ij} = dependent variables DMD, NDFD, ADFD, plasma acetate, fecal acetate, propionate and butyrate, and abundance of total bacteria, *Firmicutes*, *Bacteroidetes*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens*; μ = the mean of Y; G_i = fixed effect of group (overweight and moderate condition); and $E_{(i)j}$ = random effect of mare within group. For each model, residual plots were inspected to verify the assumption that errors followed a normal distribution with a constant variance. Data presented here includes all ten mares. Data was also analyzed with post hoc elimination of two mares, one from each group, due to low DMI intake (1.4 and 1.6% BW/day), as compared to the other mares (1.75-1.83% BW/day), due to the likelihood of a positive effect of low DMI on digestibility (data is presented in Appendix DD). Differences between groups were considered significant with $p \leq 0.05$. Data is presented as mean and range.

6.5 - Results

6.5.1 - Mares

Body weights, body condition scores, rump fat measurements, and dry matter intake (DMI) for the two groups are presented in Table 21. Mean rump fat was thicker (p=0.03) in overweight mares (2.25 cm) than moderate condition mares (1.63 cm). Dry matter intake did not differ between groups (p=0.61).

Table 21 - ***Mare body weight, BCS, and rump fat measurements taken on day 0 and 15 of the study and average dry matter intake throughout the study (d1-14).***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
BW d0 (kg)	538	511-575	561	522-611	0.352
BW d15 (kg)	532	501-575	547	490-601	0.594
BCS (1-9/9)	7.3	7.0-8.0	5.3	5.0-6.0	<0.001
BMI = BW_{kg}/H_m^2	224	202-256	218	204-229	0.576
BMI = $BW_{kg}/(H_m*L_m)$	199	183-214	194	178-205	0.541
Rump Fat (cm)	2.25	1.94-2.56	1.63	1.05-2.05	0.026
Dry matter intake (% BW)	1.75	1.57-1.83	1.71	1.43-1.81	0.610

BW = Body weight (scale); BCS = body condition score; BMI = body mass index; H = height; L = length

6.5.2 - Digestibility

Dry matter, NDF and ADF apparent digestibilities did not differ between groups (Table 22). The apparent digestible energy determined in the present study was higher (2.18-2.25 Mcal/kg DM; Table 22) than the calculated value provided by the forage laboratory (2.02Mcal/kg DM; Table 18).

Table 22 - ***Hay digestibility in overweight and moderate condition mares during d11-14 of the study.***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
DE (Mcal/kg DM)	2.25	2.12-2.46	2.18	2.05-2.26	0.37
Digestibility (%)					
DM	56.3	52.6-60.5	54.0	51.6-56.1	0.191
NDF	60.2	57.9-64.6	59.0	56.4-61.7	0.506
ADF	54.9	51.5-59.9	53.5	50.1-57.3	0.514

DM = dry matter; NDF = neutral detergent fiber; ADF = acid detergent fiber

6.5.3 - Volatile Fatty Acids

Fecal acetate, propionate, and butyrate concentrations did not differ between overweight and lean mares (Table 23). However, mean plasma acetate was higher (p=0.034) in the overweight mares (1.55 mmol/L) than the moderate condition mares (1.39 mmol/L).

Table 23 - ***Volatile fatty acid concentrations in the feces (mg/g dry feces) and plasma (mmol/L) of overweight and moderate condition mares on d 11-14 of the study.***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Fecal					
Acetate	8.28	6.06-10.75	8.31	6.43-9.57	0.973
Propionate	4.44	2.77-6.31	4.70	3.52-6.67	0.770
Butyrate	0.68	0.46-0.91	0.68	0.53-0.97	0.972
Plasma					
Acetate	1.55	1.43-1.65	1.39	1.22-1.47	0.034

6.5.4 - Fecal Bacteria

A difference in the absolute abundance of fecal total bacteria, *Firmicutes,* and *Bacteroidetes* was not detected (Table 24). Furthermore, the mean relative abundance of fecal *Firmicutes* (67.4% [51.4-88.5] and 87.3% [59.6-106.7]) and *Bacteroidetes* (26.5% [17.7-37.8] and 28.8% [22.9-33.5]) in the feces of overweight vs. moderate condition mares, respectively, did not differ (p=0.093 and 0.595, respectively).

A difference in total bacteria *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* was not detected (Table 25). Furthermore, a difference in absolute abundance (Table 26) and proportion cDNA:DNA (Table 27) for total active bacteria, active *Ruminococcus flavefaciens*, and active *Fibrobacter succinogenes* was not detected.

Table 24 - ***Abundance (log_{10} copies DNA per gram of feces) of total bacteria, Firmicutes, and Bacteroidetes in overweight vs. moderate condition mare feces.***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	9.07	8.84-9.21	9.14	8.99-9.33	0.505
Firmicutes	8.89	8.85-9.09	9.07	8.87-9.26	0.125
Bacteroidetes	8.48	8.29-8.70	8.60	8.51-8.76	0.180

*Bacterial abundance was determined by use of SYBR® Green primers (*Table 20*).*

Table 25 - ***Abundance (log_{10} copies DNA per gram of feces) of total bacteria, Ruminococcus flavefaciens, and Fibrobacter succinogenes per gram of overweight vs. moderate condition mare feces.***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	8.38	8.31-8.46	8.38	8.34-8.46	0.824
Ruminococcus flavefaciens	6.77	6.49-6.98	6.82	6.71-6.95	0.643
Fibrobacter succinogenes	5.33	5.07-5.67	5.54	5.42-5.77	0.142

*Bacterial abundance was determined by use of TaqMan® primers/probes (*Table 20*).*

Table 26 - ***Abundance (log_{10} copies cDNA per gram of feces) of active total bacteria, Ruminococcus flavefaciens, and Fibrobacter succinogenes as determined by use of TaqMan® primers/probes (*****Table 20*****).***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	6.88	6.72-7.06	7.05	6.76-7.25	0.152
Ruminococcus flavafaciens	5.13	5.02-5.31	5.35	5.03-5.72	0.131
Fibrobacter succinogenes	4.96	4.45-5.48	4.97	4.52-5.52	0.956

Table 27 - ***Proportion of active total bacteria, Ruminococcus flavefaciens, and Fibrobacter succinogenes (# copies 16S rcDNA per gram of feces / # copies 16S rDNA per gram of feces) as determined by use of TaqMan® primers/probes (*****Table 20*****).***

	Overweight Mares (n=5)		**Moderate Condition Mares** (n=5)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	3.1	2.1-4.7	4.6	2.1-7.4	0.17
Ruminococcus flavafaciens	2.5	1.1-3.9	3.8	2.3-5.9	0.14
Fibrobacter succinogenes	38.6	9.6-51.8	33.1	14.0-57.3	0.66

6.6 - Discussion

6.6.1 - Measures of Adiposity

Dugdale *et al*. (2012) evaluated BCS as a predictor for body fat mass in horses using deuterium oxide dilution and reported that % body fat and BCS had a curvilinear positive correlation. Large changes in % body fat during weight loss were associated with small changes in BCS (i.e. visceral fat changes will not be observed). However, BCS (Henneke et al., 1983) was utilized in the present study as a inexpensive, convenient, and non-invasive way to subjectively assess body fat mass. A inability to detect a difference in calculated BMI in the present study is not surprising as BMI is weakly correlated with percent fat mass ($R^2 = 0.44$) (Henneke et al., 1983) and BCS ($R^2 = 0.51$ (Henneke et al., 1983) and 0.60 (Donaldson et al., 2004), respectively). Rump fat thickness in the present study (1.05-2.56 cm) was similar to reports by Westervelt *et al.* (1976) (1.55cm) in adult horses, but higher than reported by Ragnarsson and Jansson (2011) (0.88cm) Icelandic horses. The latter is surprising to the author as the Icelandic horses had similar BCS (7/9) to the overweight mares in the present study. The difference between measurements in Ragnarsson and Jansson (2011) vs. findings in the present study may be due to morphometric differences between the horses in each study; however, a difference in technique cannot be ruled out.

6.6.2 - Digestibility

Digestible energy of forage can be determined by a digestibility trial or by calculation using the following: DE (kcal/kg DM) = 2,118+12.18(CP%)-9.37(ADF%)-3.83(hemicelluloses %)+47.18(fat %)+20.35(NSC)-26.3(ash %) (Pagan, 1998), which is only an estimate and does not represent animal factors that affect digestibility. Predicting DE using forage chemical composition appears to underestimate total daily DE intake and may lead to overfeeding of

calories. However, this discrepancy in DE determination does not explain why some horses become overweight and obese when consuming the same forage as lean horses. We chose a 4-day total collection period as a collection of this duration has been described in previous forage digestibility studies in horses (Eckert et al., 2010; Ordakowski-Burk et al., 2006); although, a standard has not been set and collection periods range from 3 days (Staniar et al., 2010) to 5 days (Crozier et al., 1997; LaCasha et al., 1999). Orchard Grass hay DM, ADF and NDF digestibility in the present study is similar to previous reports in forage-fed horses (Eckert et al., 2010; Ordakowski-Burk et al., 2006; Staniar et al., 2010). However, there are differences in forage quality used and DMI across the forage digestibility studies in horses (Table 28). Neutral detergent fiber digestibility was higher in the present study than in prior forage digestibility studies, yet NDF in the Orchard Grass hay was similar to other studies.

Table 28 - *Comparison of forage digestibility studies in horses.*

Variable	Value	Crozier *et al.* (1997)	Drogoul *et al.* (2001)	Miragla *et al.* (2006)	Ordakowski-Burk *et al.* (2006)	Edouard *et al.* (2008)	Eckert *et al.* (2010)	Staniar *et al.* (2010)	Potts *et al.* (2010)	This study
Signalment		Arabian geldings	Mini Ponies	Saddle horses	Thorough-bred geldings	Standard-bred geldings	Thorough-bred & Quarter Horses	Quarter Horse mares	Quarter Horse geldings	Mixed-breed adult mares
Hay Type		Tall Fescue	Chopped meadow hay	Unspecified	Timothy	Grass	Bermuda	Teff (early)	Bermuda	Orchard Grass
Hay Analysis	CP	11	--	8	14	12	10	11	9	15
	ADF	40	38	38	35	--	37	40	38	42
	NDF	72	70	62	62	64	73	71	62	63
DMI	% BW	2.5		--	1.8	1.7	1.8	1.7	--	1.7
Digestibility	DM	48	--	57	56	51	53	55	55	55
	ADF	37	42	43	42	--	--	55	51	54
	NDF	44	46	51	55	--	46	56	56	60

Studies were selected if conducted on adult horses fed grass forage as the total ration.

A difference in digestibility between overweight and moderate condition mares was not detected in the present study (Table 22). Vermorel, Vernet and Martin-Rosset (1997b) compared grass hay (12.6% CP, 64.1% NDF, 35.1% ADF DMB) digestibility in ponies versus horses and reported that dry matter, NDF and ADF digestibilities and digestible energy did not differ between ponies (51.2, 47.2, 48.5 and 45.7% ,respectively) and horses (51.2, 48.0, 48.6 and 46.1%, respectively). Ragnarsson and Jansson (2011) compared grass haylage digestibility in overweight (BCS 7-8/9) Icelandic horses versus Standardbred horses in ideal body condition (BCS 4-5/9). Grass was either harvested post-heading (11.1% CP, 61.1% NDF, 36.2% ADF, 18.6MJ GE/kg DM) or post-seeding (6.1% CP, 62.7% NDF, 37.7% ADF, 18.6MJ/kg DM). Mean apparent organic matter digestibility and digestible energy was higher in the Standardbred horses (56.5% and 54.0%, respectively) than the Icelandic horses (53.6% and 51.7%, respectively) when fed the less mature haylage. Higher digestible energy and OM digestibility in the Standardbred horses was surprising due to their lower BCS and higher OM intake than Icelandic horses. However, when the horses were fed the more mature haylage, an effect of breed was not detected.

Despite the inability to detect a statistical difference in digestibility between overweight and moderate condition mares; the mean difference in digestible energy between overweight and moderate condition mares may have biological relevance. A difference in 0.067Mcal/kg hay DM in the 500kg horse fed 2% BW per day in hay (10kg hay DM per day) translates to an extra 245Mcal DE per year in the overweight horse. At about 23kg per BCS and 7Mcal per kg of adipose, each BCS in a 500kg horse contains about 161Mcal. Therefore, this difference in digestible energy could account for an increase in more than 1 BCS within a single year.

6.6.3 - Volatile Fatty Acids

The fecal VFA concentrations in the present study (Table 23) were higher than concentrations in the feces of geldings limit-fed alfalfa cubes (2.84mg acetate, 0.89mg propionate, and 0.55mg butyrate per g of fecal DM) as reported by Hussein *et al.* (2004). This difference could be due to technique or could represent an effect of diet (alfalfa vs. hay) or individual variation in VFA production by hindgut microbes or VFA absorption. Argenzio, Southword and Stevens (1974) reported that total VFA concentrations varied from 20 to 60mmoles/L in the hindgut among ponies fed the same pelleted feed. Other VFAs, such as valerate, isovalerate, and isobutyrate, were not evaluated in the present study as they collectively represent less than 10% of total VFAs in horse feces (Hussein et al., 2004). Therefore, a comparison of the volatile fatty acids ratios in the present study and prior studies cannot be made.

Plasma acetate concentrations were higher in overweight mares. Possible causes for increased plasma acetate in overweight mares could be due to increased microbial VFA production, reduced microbial acetate utilization, increased VFA absorption across the colonocytes, reduced acetate oxidation, or reduced hepatic triglyceride synthesis. Cecal and colon luminal VFAs do not represent VFAs exclusively utilized by the host as VFAs present in the gut are both produced and utilized by gut microbes (Duncan et al., 2002a; Duncan et al., 2004). For example, members of the *Firmicutes* phyla, i.e. *Roseburia* spp. and *Eubacterium* spp., metabolize acetate to butyrate (Duncan et al., 2002a). Measurement of portal venous VFA concentration in conjunction with cecal and colon VFA concentrations would be a more accurate method to elucidate VFA absorption rates *in vivo* and thus determine if a difference in VFA absorption exists between overweight and moderate condition horses.

The fates of absorbed VFAs (once in the portal vein) include hepatic gluconeogenesis (propionate), beta-oxidation in hepatic or skeletal muscle tissue, and storage in adipose tissue. Therefore, venous VFA concentrations do not accurately represent concentrations of VFAs absorbed in the portal vein, particularly propionate and butyrate due to extensive metabolism in the liver and colonocytes, respectively. Of the three VFAs evaluated in the present study, only acetate in venous plasma was reported because peripheral tissues utilized acetate as an energy source (Pethick et al., 1993).

Direct measurement of VFA concentrations in the hindgut or factors such as lumen pH, lumen sodium concentration, or gut perfusion was beyond the scope of this study. Once absorbed, acetate can be oxidized via the TCA cycle or stored in the form of adipose, as acetate is the primary substrate for *de novo* fat synthesis in the horse (Suagee et al., 2010). The relationship between the overweight condition and increased plasma acetate should be further explored. Portal vein catheterization in horses was described by Baker *et al*. (1970) and can be placed in conjunction with cecal cannulas; however, both are invasive procedures.

To evaluate the role of acetate in equine obesity, acetogenic (i.e. acetyl-CoA synthase (Gagen et al., 2010; Rey et al., 2010)) and acetate-utilizing gene (i.e. butyrylCoA:acetateCoA transferase (Louis and Flint, 2007)) expression could be evaluated in the cecal and colonic contents of cannulated obese and lean horses limit fed the same grass hay diet. The author hypothesizes that overweight and obese horses have higher acetogenic gene expression and lower acetate-utilizing gene expression than lean individuals. Microbial functions are conserved across different taxonomic groups; therefore, evaluation of function provides more information on how a microbe can impact a host more than microbial identity and taxonomic classification alone. The first attempt to quantify gene expression should be performed with previously

designed primers (Gagen et al., 2010; Louis and Flint, 2007; Rey et al., 2010). Although these primers were used in the unsuccessful attempt to quantify gene expression in the overweight and moderate condition mare feces (Appendix CC), RNA is an unstable molecule (T½ of 40 sec 60min (Richards et al., 2008) and RNases are abundant in any environment. Furthermore, acetate concentrations are highest in the cecum and reduce aborad to the rectum (Argenzio et al., 1974); therefore, feces is likely not the best sample for evaluating functional genes of equine hindgut microbes. If the lack of amplification in the present study is due to poor functional gene yield in feces, then the previously published primers (Gagen et al., 2010; Rey et al., 2010) may work for evaluation of equine colon lumen contents. However, in the event that amplification is not possible after attempts to improve assay performance, primers specific to genes present in the horse would need to be designed.

6.6.4 - Equine Fecal Bacteria

The abundance of *Firmicutes* is positively associated with obesity in humans (Ley et al., 2006a; Turnbaugh et al., 2009), pigs (Guo et al., 2008b), and rodents (Ley et al., 2005; Turnbaugh et al., 2008). However, a difference in abundance of *Firmicutes* was not detected between overweight and moderate condition adult mare in the present study (Table 24). Furthermore, the ratio of fecal *Firmicutes*: *Bacteroidetes* was similar for the overweight (1.05) and moderate condition (1.06) mares.

The wide variation in the abundance of *Bacteroidetes* reported previously (Daly et al., 2001; Shepherd et al., 2012b; Willing et al., 2009) may be due to host factors, host diet, or environmental factors, which were not controlled between studies, or it could be due to the different methodologies used to evaluate gut microbes in horses (i.e. clone libraries vs. pyrosequencing). In the present study, the abundance of *Bacteroidetes* was characterized using

primers previously designed from *Bacteroidetes* sequences in the RDP database (rdp.cme.msu.edu) (De Gregoris et al., 2011).

Ruminococcus flavefaciens and *Fibrobacter succinogenes* are not the only fibrolytic bacterial species in the equine gut and this study was not designed to investigate the total fibrolytic bacterial population in equine feces. Other fibrolytic bacteria in the equine hindgut include *Clostridium* spp. and *Eubacterium* spp. (Daly et al., 2001); however, the fibrolytic population in the equine hindgut is poorly described. In order for fibrolytic bacteria to hydrolyze fiber molecules these microbes must be able to bind to and subsequently digest the fiber molecules. An enhanced understanding of how these microbes attach to and subsequently degrade fiber particles in the equine hindgut is needed in order to target this population for obesity management. Potential strategies for targeting the hindgut microbiome for obesity prevention and treatment include signaling molecules administered to the host to reduce microbial cellulolytic gene expression, agents that negatively impact the attachment of fibrolytic bacteria to fiber molecules (Jun et al., 2007), or agents that negatively impact the ability of bacteria to form biofilm in the fiber molecules (Rosewarne et al., 2011). However, before the utility of these proposed methods can be utilized, an association between hindgut microbes, specifically cellulolytic microbes, and obesity should be made.

In the mouse, presence vs. absence of gut microbes promotes the obese state through enhancing energy extraction from the host's diet (Samuel and Gordon, 2006) and promoting fat storage in the host (Bäckhed et al., 2004; Samuel and Gordon, 2006). Although evaluation of fat storage in the mares was beyond the scope of this study, digestible energy did not differ between groups.

6.7 - Acknowledgements

The present study was funded by the College of Veterinary Medicine Intramural Grant. Hay was provided by Standlee Hay Company. I am very grateful to Dr. Roderick Mackie, University of Illinois, for supplying the *Fibrobacter succinogenes* and *Ruminococcus* strains that served as standards for real-time PCR analysis. I would like to thank Stewart Milton for her assistance throughout the study, Dr. Amy Tanner and Barbara Self for their assistance in the digestibility analyses, Dr. Mark Hanigan, Tara Wiles, and Deepthi Nayananjalie for their assistance in volatile fatty acid analysis, and Dr. Mike McGilliard for statistical guidance. Special thanks goes to the twenty-six undergraduate students that assisted with horse care, sample collection, sample processing, and analysis.

Section 7 - Conclusion

From the present work, the following conclusions have been made:

1. Feeding a high (18%) vs. moderate (12%) NSC hay to Arabian geldings did not result in hyperinsulinemia. Repeating this work in insulin resistant horses or in an insulin sensitive cohort with the addition of a dynamic test (i.e. frequently sampled intravenous glucose tolerance test [FSIGT]) would further characterize the effect of a high (18%) and moderate (12%) NSC hay on insulin and glucose dynamics in adult horses. Further evaluation of the influence of hay NSC on equine insulin dynamics would help to determine appropriate hay recommendations for horses with or at risk of laminitis.

2. Fecal preparation and DNA extraction methodology influenced results from gelding fecal microbiome analysis, both with DGGE and real-time PCR analysis. Therefore, the present work emphasizes the importance of using the same methodology for comparison across samples and also emphasizes challenge in comparing results across studies. The 260/280 and 260/230 ratios in the DNA extracted from horse feces in the present study were low, suggesting high concentrations of protein and organic molecules like polysaccharides, which can inhibit DNA polymerase and subsequent DNA amplification. However, DNA quality appeared adequate based on amplification as evaluated by agarose gel band quality and real-time PCR results. Further elimination of PCR polymerase inhibitors, by improvement in or added inhibitor removal steps during DNA extraction, may augment DNA amplification and change the results from fecal microbial analyses.

3. Feeding high (18% NSC) vs. moderate (12% NSC) hay did not influence gelding fecal microbiomes as evaluated with DGGE. However, large variation between individuals on the same hay was observed. Perhaps if dietary NSC concentration differed by more than 6% (i.e.

hay vs. hay + grain diet), or additional nutrients were available, fecal DGGE banding patterns would cluster by diet. To specifically evaluate the effect of levan-type fructans on the equine hindgut microbiome, fecal DGGE banding patterns could be compared between two groups of horses fed the same grass hay diet plus an oral bolus of levan-type fructans administered to one group and an oral bolus of water administered to a control group.

4. Time and day influenced the fecal microbiome of the geldings as evaluated with DGGE. The present author concludes that timing of fecal sample collection is critical when evaluating small changes in the equine fecal microbiome and comparisons between horses should be taken at the same time on the same day. Ideally samples should be combined within and between days (combining AM and PM samples collected over a 4-day period) to eliminate the influence of time and day on data analysis.

5. The present study was the first to quantify the abundance of *Firmicutes* and *Bacteroidetes* phyla with real-time PCR, a more sensitive method for determining bacterial abundance than previously used in the horse (i.e. clone library generation and FISH). The fecal microbiome in adult horses fed Orchard Grass hay is dominated by *Firmicutes* phyla members and *Bacteroidetes* members represent a smaller portion of the fecal microbiome than found in humans. Although the abundance of *Firmicutes* was higher in geldings fed the 12% vs. 18% NSC hay, the significance of these findings is unclear because of the small number of horses evaluated and because the difference was detected with only one of the three previously published *Firmicutes* primer sets evaluated. However, due to the high abundance of *Firmicutes* in equine feces, this phylum should be further evaluated with high through-put sequencing methodology to characterize bacterial changes within the *Firmicutes* phyla (i.e. evaluating

changes in the relative abundance of *Clostridium* spp.) in the feces of horses fed hays of differing NSC concentration.

6. Thirty-eight percent of bacterial sequences identified from the pyrosequencing work in Section 5 were unable to be identified; therefore, the richness of the equine fecal microbiome is likely higher than reported in the present work. Equine fecal bacterial classification may be improved by sequencing longer reads; however, due to the large proportion of uncultivable bacteria in the gut, bacterial identification is limited by the databases of sequences available (i.e. RDP and NCBI). Submission of sequences from newly characterized bacteria will improve sequence identification of bacteria present in the equine fecal microbiome.

7. The numerically higher diet apparent digestibility in overweight vs. moderate condition mares is of biological relevance and could contribute to a gain in BCS within a year's time. To detect a 2% difference in hay DM digestibility in adult mares, this work should be repeated with 14 obese and 14 lean adult mares.

8. Plasma acetate was higher in the overweight vs. moderate condition mare group. The exact cause and significance of higher plasma acetate was beyond the scope of the study. However, higher plasma acetate is suggestive of higher microbial acetate production, higher acetate absorption, or reduced acetate metabolism. The significance of the higher plasma acetate in the overweigh mares warrants further investigation as acetate is the predominate substrate for *de novo* lipogenesis in the horse. Quantification of acetate concentration in the cecum and portal vein of adult lean (BCS 4-5/5) vs. obese (BCS 8-9/9 horses would better characterize the cause of higher plasma acetate in the overweight mares.

9. Fecal samples of horses do not appear to be adequate for evaluation of hindgut bacterial gene expression. To further explore microbial gene expression by equine hindgut

microbes, the same cellulolytic and acetogenic gene primers described in Appendix CC could be used to quantify functional microbial gene expression in equine cecal samples. Presence of higher cellulolytic and acetogenic gene expression in obese vs. lean adult horses would be suggestive that higher hindgut microbial acetate production is the cause for higher plasma acetate in the overweight adult mares in the present study.

Section 8 - Appendices

Appendices to Section 3

Appendix A – Arabian gelding signalment

Horse ID	Age (year)	8/28/2008 BW (kg)	8/28/2008 BCS (1-9)	8/28/2008 Plasma Insulin (µIU/ml)	8/28/2008 ACTH (pg/ml)
229	17	491	6	29.8	26.4
231	14	446	8	23.8	72.6
232	22	484	6	39.9	128.0
241	11	496	9	43.7	38.4
244	17	636	9	48.8	33.8
248	19	468	6	49.0	58.9
249	9	468	7	30.7	130.0
250	15	418	6	11.2	54.7

Samples collected 8/28/2008 were taken when geldings were out on a cool-season pasture in Middleburg, VA.

Appendix B – Daily Temperatures at the VMRCVM Research barn

Period	Dates	Maximum (°C)	Minimum (°C)
Acclimation	10/24/2008 – 11/23/2008	11.7 (22.8 - ¯2.2)	¯0.8 (9.4 - ¯10.6)
1	11/24/2008 – 12/22/2008	7.5 (17.2 - ¯2.8)	¯3.8 (7.2 - ¯13.9)
Wash-out	12/23/2008 – 1/18/2009	6.3 (19.4 - ¯8.9)	¯5.7 (3.9 - ¯21.1)
2	1/19/2009 – 2/16/2009	7.4 (20.0 - ¯6.7)	¯5.8 (8.3 - ¯15.6)

Data is reported as mean (range).

Appendix C – Guaranteed Analysis and Composition of EquiMin Granular

Nutrient		
Calcium	(min)	16.0%
Calcium	(max)	18.0%
Phosphorus	(min)	8.0%
Salt	(min)	22.0%
Salt	(max)	26.0%
Magnesium	(min)	1.0%
Sulfur	(min)	0.3%
Potassium	(min)	0.6%
Iodine	(min)	30 ppm
Copper	(min)	620 ppm
Cobalt	(min)	6 ppm
Selenium	(min)	16 ppm
Zinc	(min)	2,450 ppm
Manganese	(min)	1,250 ppm
Vitamin A	(min)	200,000 IU/lb
Vitamin B	(min)	20,000 IU/lb
Vitamin E	(min)	1,000 IU/lb

Southern States, Richmond, VA

Ingredients:

Monodicalcium-Dicalcium Phosphate, Defluorinated Phosphate, Salt, Magnesium Oxide, Potassium Chloride, Potassium Sulfate, Magnesium Sulfate, Calcium Carbonate, Potassium Iodide, Manganous Oxide, Manganese Proteinate, Zinc Oxide, Zinc Proteinate, Ferrous Sulfate, Copper Sulfate, Copper Proteinate, Cobalt Carbonate, Sodium Selenite, Iron Oxide (coloring agent), Vitamin A Supplement, Vitamin D3 Supplement, Vitamin E Supplement, Menadione Dimethylpyrimidinol Bisulfite, Riboflavin Supplement, Niacin Supplement, Calcium Pantothenate, Vitamin B12 Supplement, Choline Chloride, d-Biotin, Thiamine Mononitrate, Yeast Culture, Yeast Fermentation Solubles, Petrolatum, Mineral Oil, Montmorilinite Clays, Natural and Artificial Flavors Added, Molasses Products, Processed Grain By-Products, Roughage Products.

Appendix D - Coat-A-Count Insulin Radioimmunoassay

Concept

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

Materials

a. Borosilicate heavy wall 12x75mm disposable culture tubes (glass) (Fisher 14-958-10B)
b. Coat-A-Count Insulin Radioimmunoassay (RIA) (PITKIN-5, Siemens, Los Angeles, CA)
c. Deionized water
d. 200µL of serum per sample

Allow all reagents to come to room temperature before beginning.

Methods

1. Label tubes
    a. 4 uncoated tubes and non-specific binding (NSB) tubes in duplicate
    b. 14 insulin antibody-coated tubes A (maximum binding) through G in duplicate

Calibrator	IRP (µIU/ml)
A (maximum binding)	0
B	5.7
C	16
D	51
E	102
F	201
G	389

   c. Additional antibody-coated tubes (in duplicate) for controls and samples

2. Pipet 200µL of the zero calibrator A into the NSB and A tubes, and 200µL of each remaining calibrator, control, and patient sample into the tubes prepared. Pipet directly into the bottom of the tube.
3. Add 1.0ml of ^{125}I Insulin to every tube and vortex. Complete this step within 40 minutes of step 2. T tubes require no further processing until step 6.
4. Cover tubes with Para film and incubate for 18-24hr at room temperature (15-28°C).
5. Decant thoroughly; remove all visible moisture to enhance precision. Allow tubes to drain for 2-3 minutes then strike tubes sharply on absorbent paper to shake off all residual droplets. Do not decant T tubes.
6. Count for 1 minute in a gamma camera.

Calculation of Results

1. To calculate insulin concentrations from a logit-log representation fo the calibration curve, first calculate for each pair of tubes the average NSB-corrected counts per minute
   a. Net Counts = (Average Counts Per Minute [CPM] – Average NSB CPM) x 100
2. Determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%
   a. Percent Bound = (Net Counts / Net MB Counts) x 100
3. Plot Percent Bound (Y axis) by Concentration (X axis) for each of the nonzero calibrators to determine your calibration curve.

a. T = Total Counts (counts per minute)
b. % NSB = 100 x (Average NSB Counts / Total Counts)
c. % MB = 100 x (Net Counts / Total Counts)

Validation

A radioimmunoassay (RIA) was first validated for use in measuring equine serum insulin concentrations by Reimers *et al.* (1982). Reimers *et al.* (1982) validated the insulin RIA against measurements of insulin extracted from equine pancreas measured by gas chromatography. The coefficient of variation (CV) and sensitivity for equine serum insulin measurements was reported as 4.4-10.7% and 3.2uIU/ml, respectively. Borer *et al.* (2012) reported that the Siemens Coat-A-Count insulin RIA was also validated at the Clinical Pathology Laboratory, Royal Veterinary College with an intra and inter-assay CV of 6.5+/-5.1 and 7.4+/-4.3%, respectively.

Currently there are two immunoassays marketed for use in horses: Mercodia Equine Insulin Enzyme-Linked Immunosorbent Assay (ELISA), and Shibayagi Equine Insulin ELISA, which both use porcine insulin monoclonal antibodies (Tinsworth *et al.* 2011). Timsworth *et al.* (2011) evaluated the utility of 6 commercial immunoassay kits, including the previously mentioned kits marketed for horses and the Siemens Coat-A-Count RIA for evaluation of equine plasma insulin validated against liquid chromatography-mass spectrometry (LCMS). Timsworth *et al. (*2011) concluded that the Siemens Coat-A-Count RIA kit was the most accurate method for determination of equine plasma insulin concentrations. However, authors (Timsworth 2009 and 2011) pointed out that standards should be diluted with charcoal-stripped plasma as compared to water when measuring insulin concentrations above 30ng insulin/mL as dilution with water resulted in overestimation of plasma samples with high insulin concentrations (i.e. in hyperinsulinemic horses). All Arabian geldings in our study were normoinsulinemic throughout the study and therefore, sample dilution was unnecessary, support for the use of the standard manufacturer's protocol. Borer *et al.* (2012) recommended dilution of samples with insulin-deplete serum resulted in more accurate results than when the manufacturers dilution was used.

We measured insulin concentrations in Arabian gelding plasma using the Coat-A-Count Insulin RIA and reported an intra-assay CV of 1.7%, which is lower than previously reported (8.1% (Tinworth et al., 2009) and 9.7% (Tinworth et al., 2011)).

Table 29 – Comparison of plasma insulin concentrations between serum insulin analysis using the Coat-A-Count RIA vs. two commercial labs.

Horse ID	Sampling Date	Plasma Insulin (µIU/ml)		
		RIA	Cornell	Michigan
229	12/1/2008	3.0	7.7	7.1
232	12/8/2008	27.4	32.2	43.1
248	2/2/2009	62.4	50.3	71.9
250	2/16/2009	2.3	6.4	4.2

Appendix E - Multi-Species Leptin Radioimmunoassay

Concept

See RIA concept in Appendix A.4. The Millipore Multi-Species Leptin assay utilizes 125I-labeled Human Leptin and a Multi-Species Leptin antiserum to determine the level of Leptin in serum, plasma or tissue culture media by the double antibody/PEG technique.

Materials (Supplied)

Multi-Species Leptin Radioimmunoassay (RIA) (#XL-85K, Millipore, Billerica, MA)

A. Assay Buffer

- 0.05M Phosphosaline, pH 7.4, containing 0.025M EDTA, 0.08% Sodium Azide, 0.05% Triton X-100, and 1% RIA Grade BSA
- Quantity: 40 mL/vial
- Preparation: Ready to use

B. Antiserum

- Guinea Pig anti-Multi-Species Leptin Antibody in Assay Buffer
- Quantity: 26 mL/vial
- Preparation: Ready to use

C. 125I-Human Leptin 125

- I-Human Leptin Label, HPLC purified (specific activity 135 Ci/g).
- Lyophilized for stability. Freshly iodinated label contains <3 µCi, (<111 kBq) calibrated to the 1
- St Monday of each month.
- Quantity: 27 mL/vial upon hydration
- Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing.

D. Label Hydrating Buffer

Assay Buffer containing Normal Guinea Pig IgG as a carrier. Used to hydrate

125

I-Human Leptin

Quantity: 27 mL/vial

Preparation: Ready to use

E. Standards

- Purified Recombinant Human Leptin in Assay Buffer at the following concentrations:1, 2, 5, 10, 20, 50 ng/mL.
    - Since the Multi Species Leptin antibody was raised against Human Leptin, Human Leptin standards are used in this assay. The cross-reactivity of this antibody to leptin molecules of many other species is unknown. Therefore, it is recommended that investigators use ng/mL Human Equivalent (ng/mL HE) as the unit of measure.
    - Quantity: 1mL/vial
    - Preparation: Ready to use

F. Quality Controls 1 and 2

- Purified Recombinant Human Leptin in Assay Buffer
    - Quantity: 1 mL each
    - Preparation: Ready to use

G. Precipitating Reagent

- Goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide
  Quantity: 260 mL/vial
  Preparation: Ready to use; chill to 4°C.

Materials

1. Borosilicate glass tubes, 12 x 75 mm. NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.
2. 100 µL pipet with disposable tips
3. 100 µL and 1.0 mL repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (Use of fixed-angle buckets are not recommended.)
5. Absorbent paper
6. Vortex mixer

7. Refrigerator
8. Gamma Counter

Method

For optimal results, accurate pipetting and adherence to the protocol are recommended.

A. Assay Set-Up, Day One
   i. Pipet 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µl to the Reference (Bo) tubes (5-6), and 100 µL to tubes 7 through the end of the assay.
   ii. Pipet 100 µL of Standards and Quality Controls in duplicate (see Assay Procedure Flow Chart).
   iii. Pipet 100 µL of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when leptin concentrations are anticipated to be elevated or when sample size is limited. Additional AssayBuffer must be added to compensate for the difference so the volume is equivalent to 100 µl, e.g., when using 50 µL sample, add 50 µL Assay Buffer.)
   iv. Pipet 100 µL of Multi Species Leptin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
   v. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

B. Day Two
   i. Pipet 100 µL of ^{125}I-Human Leptin to all tubes.
   ii. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

C. Day Three
   i. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
   ii. Vortex and incubate 20 minutes at 4°C.
   iii. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, the time of centrifugation must be increased to obtain a firmer, more stable pellet (e.g., 40 minutes). A swingbucket rotor is recommended. Conversion of rpm to xg: $xg = (1.11 \times 10\text{-}5)\ (r)\ (rpm)^2$ where r =

radial distance in cm (from axis of rotation to the bottom of the tube); rpm = rotational velocity of the rotor

iv. Immediately decant the supernatant from all tubes except Total Count tubes (1-2), drain tubes for 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.

v. Count all tubes in a gamma counter for 1 minute. Calculate the ng/mL HE of leptin in unknown samples using automated data reduction procedures (see the following "Calculations" section).

Appendix F - SAS Code for GLIMMIX Procedure for Evaluating the Effect of Hay on Arabian Gelding Insulin, Glucose, Leptin and Triglycerides.

```
dm 'clear log'; dm 'clear output';
data cervtables1; set megan.cervtables;
proc print;

ods graphics on;
ods rtf;

proc glimmix data=cervtables1;
        class ID period hay;
        model  Wt___kg_ = Period Period|Hay/ddfm=kr;
        random Id;
        lsmeans Period*Hay/slice=(Hay Period) plot=mean(sliceby=Hay join);
  lsmeans Period*Hay/cl diff pdiff adjust=tukey slicediff =
  (Hay Period);

  nloptions tech =nrridg;
run;

proc glimmix data=cervtables1;
        class ID period hay;
        model  Intake____offered_ = Period Period|Hay/ddfm=kr;
        random Id;
        lsmeans Period*Hay/slice=(Hay Period) plot=mean(sliceby=Hay join);
  lsmeans Period*Hay/cl diff pdiff adjust=tukey slicediff =
  (Hay Period);

  nloptions tech =nrridg;
run;

proc glimmix data=cervtables1;
        class ID period hay;
        model  Consumption__kcal_DE_kg_BW_ = Period Period|Hay/ddfm=kr;
        random Id;
        lsmeans Period*Hay/slice=(Hay Period) plot=mean(sliceby=Hay join);
  lsmeans Period*Hay/cl diff pdiff adjust=tukey slicediff =
  (Hay Period);

  nloptions tech =nrridg;
run;

proc glimmix data=cervtables1;
        class ID period hay;
        model  Ave_Intake__kg_Day_ = Period Period|Hay/ddfm=kr;
        random Id;
        lsmeans Period*Hay/slice=(Hay Period) plot=mean(sliceby=Hay join);
  lsmeans Period*Hay/cl diff pdiff adjust=tukey slicediff =
  (Hay Period);

  nloptions tech =nrridg;
run;
```

```
proc glimmix data=cervtables1;
        class ID period hay;
        model  Fructan__g_kg_BW_day_ = Period Period|Hay/ddfm=kr;
        random Id;
        lsmeans Period*Hay/slice=(Hay Period) plot=mean(sliceby=Hay join);
   lsmeans Period*Hay/cl diff pdiff adjust=tukey slicediff =
   (Hay Period);

   nloptions tech =nrridg;
run;

ods rtf close;
ods graphics off;
```

Appendix G – Expansion of Discussion from Section 3

Strict recommendations for feeding a <10% NSC hay to horses at risk of laminitis (Frank et al., 2010) may not be necessary since feeding an 18% NSC hay did not cause hyperinsulinemia in overweight Arabian geldings. Although none of the horse developed hyperinsulinemia, dynamic evaluation of insulin and glucose regulation was beyond the scope of the study. Hyperinsulinemia serves as a marker for insulin resistance; however, not all insulin resistant horses are hyperinsulinemic. Furthermore, all analyses were performed on fasted blood samples and the glycemic response to the high (18%) vs. moderate (12%) NSC hay was not evaluated. Borgia *et al.* (2011) reported that mean peak insulin, but not glucose, concentrations were higher (59.9uU/ml) in healthy Quarter Horses fed a 17% NSC (DMB) as compared to a 4% NSC hay (17.7uU/ml). Peak insulin concentrations occurred at a mean 111 minutes for both hay groups. However, in horses with polysaccharide storage myopathy, a condition associated with enhanced insulin sensitivity, the peak insulin did not differ between hay groups. While peak insulin concentrations exceeded the normal range for non-fasted horses fed forage (>40µIU/L, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY); insulin concentrations returned to the normal range by 150minutes post-meal. Furthermore, the difference in the fructan content between the 17% (3.3% fructan DM) vs. 4% NSC (2.1% fructan DM) was much smaller (difference of only 1.2% DM) as compared to the ten-times higher ESC concentration in the 17% NSC hay than the 4% NSC hay. Borer et al. (2012) reported higher median and area under the curve insulin concentrations in previously laminitic adult ponies fed pasture and hay in the fall as compared to the spring. The ponies were fed 1g/kg glucose, fructose, or inulin once daily with 500g of a high fiber (25% AF), low starch (4% AF) commercial feed for three days; peak insulin concentrations increased only in the never

laminitic ponies. Therefore, the role of fructan on insulin sensitivity and hyperinsulinemia has not been fully elucidated and should be further investigated.

Appendices to Section 4

Appendix H - Standards for Real-Time PCR

Bacterial Species Used as Standards

Escherichia coli

- Genome size = 4,639,675 (NCBI accession # U00096)
- Number of 16S rRNA gene copies = 7 (http://rrndb.mmg.msu.edu/index.php)
- Log copies at 100ng DNA/µL = 8.06

Exiguobacterium

- Genome size = 2,999,895 (NCBI accession # NC_012673.1)
- Number of 16S rRNA gene copies = 9 (http://rrndb.mmg.msu.edu/index.php)
- Log copies at 100ng DNA/µL = 8.44

Flavobacterium

- Genome size = 2,861,988 (NCBI accession # NC_009613.1)
- Number of 16S rRNA gene copies = 6 (http://rrndb.mmg.msu.edu/index.php)
- Log copies at 100ng DNA/µL = 8.282

Ruminococcus flavefaciens

- Genome size = 4,576,399bp (Miller et al., 2009)
- Number of 16S rRNA gene copies = 1 (Miller et al., 2009)
- Log copies at 100ng DNA/µL = 7.30

Fibrobacter succinogenes

- Genome size = 3,843,004 (NCBI accession # NC_017448)
- Number of 16S rRNA gene copies = 3 (http://rrndb.mmg.msu.edu/index.php)
- Log copies at 100ng DNA/µL = 7.85

Exiguobacterium Culture and DNA Extraction

Due to difficulty obtaining an adequate standard curve for the *Exiguobacterium* standard for *Firmicutes* primers, two culture methods (1/2 X trypticase soy broth [TSB] and R2A solid media) and two extraction methods (Gentra PureGene® Kit [Appendix B.3], with and without supplemental bead beating, and CTAB (Appendix I) were used to determine which method would result in the highest DNA yield and best performance in qPCR reactions.

Exiguobacterium was cultured from a glycerol stock into ½ TSB broth culture (15g TSB (Difco 2014-04-30)/L deionized water) at 37°C aerobically overnight. Ten µL of the initial culture was transferred to a fresh ½ TSB stock and allowed to grow for 1 hour prior to DNA extraction. A second 10µL of the initial overnight culture was plated onto R2A solid agar (18.2g R2A (Difco 2013-12-31)/1L deionized water) and allowed to grow aerobically overnight at 37°C prior to DNA extraction.

1ml of the ½ X TSB culture was collected, centrifuged, supernatant discarded, and pellet used for DNA extraction using the extraction techniques described below.

The concentration of *Exiguobacterium* DNA extracted using the CTAB protocol was lower (20-32ng/µL) than when extracted using the Gentra PureGene® Kit (63-145ng/µL). Furthermore, *Exiguobacterium* DNA concentrations were higher when extracted from colonies harvested from overnight R2A media (87-145ng/µL) than from cells harvested from overnight 1/2X TSB broth (63ng/µL). *Exiguobacterium* DNA yields were also higher when the bead beating step was added to the Gentra PureGene® Kit step #8 (Appendix B.3). Serial dilutions of *Exiguobacterium* DNA extracted using the Gentra PureGene® Kit G+ protocol from both R2A broth and 1/2X TSB broth amplified equally (efficiency goal of 90-100%); therefore, both culture conditions were considered adequate provided DNA was extracted using the Gentra PureGene® kit with added bead beating. *Exiguobacterium* DNA was applied as a standard for the quantification of *Firmicutes* abundance determination only as freshly extracted DNA as a single freeze/thaw cycle resulted in poor amplification efficiency (<90% efficiency), even when DNA was thawed at 4°C for 4-8hr.

Appendix I - CTAB DNA Extraction Protocol

Modified from

http://www.mbari.org/phyto-genome/pdfs/CTAB%20DNA%20Extract_GenomeSeq%20quality.pdf

and Phyllis 3 pg 54

Materials

1. CTAB buffer
    a. 2% CTAB (hexadecyltrimethylammonium bromide)
    b. 100 mM TrisHCl [pH=8]
    c. 20 mM EDTA,
    d. 1.4 M NaCl
    e. 0.2% β-mercaptoethanol [added just before use]
    f. 0.1 mg/mL proteinase K [added just before use])
2. TE Buffer (Gaby 7/13/09)
    a. 100mM Tris
    b. 100mM EDTA
    c. pH 8.03

Method

1. Obtain ¼ plate culture (24hr culture) → 1ml PBS solution in a 1.5ml tube, vortex
2. Centrifuge 15,000g 1 min, discard supernatant
3. Resuspend pellet (≤0.17g) in 564µL TE buffer

- Add 10ug lysozyme (crystalline; stored at -20°C) to cell suspension

Invert

Incubate 37’ C 60min

Lysozyme - Fisher 066547

- Add 6µL Proteinase K (10mg/ml; stored at 4°C) and 30µL SDS (10%)

Invert (do NOT vortex)

Incubate 37°C until clear and viscous

Proteinase K - Fisher 067477

- Add 100µL NaCl (5M), invert,

Incubate 65°C 2 min

- Add 80µL pre-heated (60°C) CTAB/NaCl (10% CTAB in 0.7M NaCl)

Invert

Incubate 65°C 10 min

NOW USE WIDE BORE PIPET TIPS (ENDS REMOVED)

- Add 800µL chloroform/isoamyl alcohol (24:1) solution (stored at 4°C; Fisher BP 1752; work in the fume hood)

Gently invert 2min

Centrifuge 3,000g 5min 4°C

Transfer upper (aqueous) phase (contains nucleic acids) into new 1.5ml tube

Removes polysaccharides

- Add 800µL phenol/chloroform/isoamyl alcohol (25:24:1; Fisher BP1752; stored at 4°C)

Centrifuge 15,000g 5min

Transfer upper/aqueous phase into new 1.5ml tube

Removes protein

- Add 800µL chloroform/isoamyl alcohol (24:1; Amresco X205)

Centrifuge 10,000g 5min

Transfer upper phase to new 1.5ml tube

Removes remaining polysaccharide

- Add 0.7 volumes (560µL) isopropanol to ppt nucleic acids

Incubate at room temperature 15min

Centrifuge 12,000g 15min → should see pellet

- Add 500µL 70%EtOH (-20°C)

Invert

Centrifuge 12,000g 15-30min room temperature

Air dry pellet 15min

- Resuspend in 50µL TE buffer

Incubate 37°C 1hr

- Store at -80°C

Appendix J - Gentra PureGene® Kit G+ and G- Protocol

Materials

1. 1X PBS = 4ml 10X PBS (Fisher BP399) in 40ml deionized water
2. Gentra PureGene® Kit (Qiagen #158567, Qiagen, Valencia, CA)

Method - DNA Purification from Gram-Positive and Gram-Negative Bacteria

1. Collect the sample
    a. If using broth culture - collect 1000 µl broth culture, centrifuge at 15,000 x g for 1 minute to pellet the cells, discard the supernatant. Add 1000 µl PBS and wash the cells by resuspending the cells by pipet mixing and vortexing.
    b. If using solid media culture, transfer ¼ of the colonies on the plate to 1000 µl PBS and vortex.
2. Centrifuge for 15,000 x g for 1 min (G+ [i.e. *Exiguobacterium* and *Ruminococcus flavafaciens]*) or 30 sec (G- [*E.coli* and *Fibrobacter succinogenes*]) to pellet cells.
3. Carefully discard the supernatant by pipetting or pouring.

Continue with steps 4-7 for G+ only; if extracting G- DNA, proceed to step 9

4. Add 300 µl Cell Suspension Solution, and pipet up and down.
5. Add 1.5 µl Lytic Enzyme Solution (sored at 4°C), and mix by inverting 25 times. Incubate for 30 min at 37°C.
6. Centrifuge for 1 min at 15,000 x g to pellet cells.
7. Carefully discard the supernatant with a pipette.

Continue for both G+ and G- bacteria

8. Add 300 µl Cell Lysis Solution, and pipet up and down to lyse the cells.
    a. G+ bacteria
        i. Transfer cells and Cell Lysis Solution to sterile 0.5mm beads (Zymo Research BashingBead™ Lysis Tubes #S6002)
        ii. Vortex 5min
        iii. Centrifuge 15,000 x g for 1 minute
    b. G- bacteria
        i. Incubation for 5 min at 80°C

9. Add 1.5 µl RNase A Solution, and mix by inverting 25 times.
    a. G+ bacteria
        i. Incubate for 15min at 37°C.
    b. G- bacteria
        i. Incubate for 10min at 37°C.
10. Incubate for 1 min on ice to quickly cool the sample.
11. Add 100 µl Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.
    a. G+ bacteria
        i. Incubate on ice for 15 min
    b. G- bacteria
        i. Incubate on ice for 5 min
12. Centrifuge for 3 min at 15,000 x g. The precipitated proteins should form a tight pellet.
13. Pipet 300 µl isopropanol into a clean 1.5 ml microcentrifuge tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.
14. Mix by inverting gently 50 times.
15. Centrifuge for 3 min at 15,000 x g. The DNA will be visible as a small white pellet.
16. Carefully discard the supernatant; drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
17. Add 300 µl of 70% ethanol and invert several times to wash the DNA pellet.
18. Centrifuge for 1 min at 15,000 x g.
19. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min.
20. Add 50 µl RNase free water and vortex 5 s at medium speed to mix.
21. Incubate at 65°C for 1 h to dissolve the DNA.
22. Incubate at room temperature (15–25°C) overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage.

Appendix K - Zymo Research Soil Microbe MiniPrep™ Kit Protocol

Concept

To extract DNA from fecal bacteria, bacteria cells are lysed using a physical bead-beading method. DNA is filtered from large organic debris (i.e. fiber particles) then bound to a polypropylene membrane and washed to remove polymerase inhibitors (i.e. polysaccharides, humic, etc.). Polypropylene filters with a silica-based matrix, are superior for polysaccharide elimination during fecal bacterial DNA extraction (Cavallini et al., 2000). DNA binds to the solid membrane due to the low pH and salt in the was buffers. DNA is eluted from the polypropylene membrane and filtered to remove residual polymerase inhibitors (i.e. proteins, polysaccharides, humic molecules).

Materials

1. ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research #D6001)
2. Microcentrifuge
3. Vortex
4. Vortex adaptor (Ambion AM 10024)

Method

- For optimal performance, add beta-mercaptoethanol (user supplied) to the Soil DNA Binding Buffer to a final dilution of 0.5%(v/v) i.e., 500 µl per 100 ml.
- Before Starting: (soil samples only) Zymo-Spin™ IV-HRC Spin Filters (green tops) need to be prepared prior to use by: 1) snapping off the base, 2), inserting into a Collection Tube, and 3), spinning in a microcentrifuge at exactly 8,000 x g for 3 minutes. IF THE IV-HRC FILTER IS DRY, discard or add 500µl nuclease-free water prior to preparing.

1. Add up to 0.25 grams of soil sample to a ZR BashingBead™ Lysis Tube (0.7ml of 0.5mm lysis matrix). Add 750 µl Lysis Solution to the tube.
2. Secure on a vortex and vortex at maximum speed for 10 min.
3. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10,000 x g for 1 minute.

4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) (polypropylene silica-based 10-20µm pore size membrane; binds 5µg DNA/RNA) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.
5. Add 1,200 µl of Soil DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.
6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.
9. Add 500 µl Soil DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.
10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 50 µl DNA Elution Buffer directly to the column matrix.
11. Incubate at room temperature for 5 minutes.
12. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.
13. Transfer the eluted DNA from Step 10 to a prepared Zymo-Spin™ IV-HRC Spin Filter (green top) (10-20µm poor size polypropylene column) (see above) in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 8,000 x g for 1 minute. Store extracted DNA at -80°C.

Appendix L - Denaturing Gradient Gel Electrophoresis

Day 1 – DGGE Solution Preparation

Note, for solutions and TAE 1X buffer, use only the double distilled water from Alexander lab stored in the glass carboy by the microwave.

Label two brown bottles and two ~100ml beakers as "30" or "60". Fill a third beaker with DDI water.

To each of two 100ml beakers, add:

Item	35%	60%	Cat #
Urea	7.35g	12.6g	Fisher U15-500
40%Acrylamide/Bis (37.5:1)*	10ml	10ml	BioRad 161-0148
TAE 50X	1ml	1ml	Fisher BP13324
Formamide*	7ml	12ml	FisherBP 228-100
DDI Water	~20ml	~20ml	

* Stored @ 4°C

Add the Acrylamide:Bis, TAE 50X, Formamide, Urea, ~ 10ml DDI water and stir bar.
Set the two beakers on stir plate to dissolve urea.
Once the urea is fully dissolved, transfer first the the 30% solution to a 50ml volumetric flask.
Using a pipette, SLOWLY add DDI Water to 50ml mark.
Transfer 30% solution to a brown bottle labeled 30% Denaturing Solution w/ the date.
Repeat with 60% solution, transfer to brown bottle labeled 60% Denaturing Solution
Note, try not to introduce air while making these solutions.
Store solutions at 4°C; solutions are good for ~1month

Day 1 - DGGE Setup

Pull 30% and 60% acrylamide solutions from 4°C and set out on counter

Pull out and set up DGGE hardware

- DGGE reservoir/tub and frame
- Casting equipment
    - 2 tall and 2 short glass plates
    - 2 bracket sets
    - 2 white gasket sets
    - 2 grey gaskets
    - 2 combs
    - Casting base
    - Casting wheel
    - 2 x 30cc syringes w/ cuffs and holders
    - 16g needle and tubing
    - 2 x 50ml centrifuge tubes
    - Pipette and tips for 144 and 14.4L volumes
    - 10ml pipette tip
- Paper strips

Assemble DGGE plates, start with large plates and lay spacers along edge with notch facing the middle of the plate.

Place the short plate on top, add brackets and ensure that the edges of the glass and spacers seats snugly against the brackets and the bottom of the plates/spacers is flush with the bottom of the bracket.

It is important that the bottom of the glasses and white gaskets are flush.

Tighten brackets and slide the spacer into the space between the white gaskets, adjust until the spacer fits.

Set the casting apparatus (glass plates + bracket) in the white casting stand with the grey gasket seated under the casting apparatus.

Lock the unit in place; the plates are ready to accept the polyacrylamide solutions.

Preparation of 10% Ammonium Persulfate

To a 1.5ml tube add:

- 0.1g Ammonium Persulfate (weigh in in the 1.5ml tube)
- 1ml DDI water
- Vortex until dissolved
- Store at -20°C for 1 week, cover in aluminum foil

Casting Gels

Label syringes and centrifuge tubes high/60 and low/30 and connect the cuffs and holders. Assemble the plastic tubes to the syringes and check that the "y" piece and 16g needle are connected to the third tube.

Set the 2 50ml centrifuge tubes in the styrofoam tray.
Transfer 16ml of 30% solution into the low/30 centrifuge tube
Transfer 16ml of 60% solution into the high/60 centrifuge tube

Check that everything is assembled and ready to transfer the acrylamide solutions for polymerization.

In both the 60 and 30 centrifuge tubes add (do not splash or introduce air!):
144ul Ammonium Persulfate
14.4ul TEMED
Now, work fast!

Invert gently a 3-5 times
With the tubes attached, draw the liquid from the 30 tube to the low/30 syringe and liquid from the 60 tube to the high/60 syringe, it can be tricky to get the end of the tubes to the bottom of the centrifuge tubes.
Remove air from the syringes, but keep equal amounts of air in the tubes.

Attach syringes to the casting wheel; make sure you have the low/30 and high/60 solution on the correct side of the wheel (see "top" filling instructions on wheel). Attach the third tube, using the "y" to the syringe tubes.
Place the 16g needle at midline of the top of the two glass plates and start gently turning the wheel until the solutions rise to the top of the short plate.
Place comb. Transfer residual solutions from syringes back to one centrifuge.
Quickly rinse syringes, tubing, and needle in warm water following with DI water. Monitor the residual solution for signs of polymerization. Tubes, syringes, etc can be reused after thorough rinsing and drying.

While the DGGE gel is setting up (~1hr)…

(1) Get ice, then remove PCR products from the -20°C and place on ice.
(2) Set up the TAE 1X

Start warming up water. Fill a 2000ml flask with DDI water from the glass carboy and heat on hot plate.
Add 140ml TAE 50X to the DGGE tub.
Meanwhile, start microwaving water to make a total final volume of 7L in the DGGE tub. Do so by microwaving two 500ml flasks filled with DDI water from the glass carboy. Microwave each set at 10 minutes, it will boil. Pour hot DI water into DGGE tub. If you have to get DI water from the Alexander lab, turn on the pump, then open the spout, let water run until the number reaches 17.8-18. Fill up the glass carboy with this DI water.

Sample Prep

Prepare PCR products by adding 15µL DGGE loading dye to each PCR tube. Mix with pipet and centrifuge briefly. Note, you can store the PCR products at -20°C for up to 1 year without DGGE dye or up to 1 month with DGGE dye.

Assembling gels on DGGE frame

Once gel is set up, gently remove comb. Remove excess gel along the tall plate with a metal spatula. Place paper strips into gel wells to dry.

Remove gel apparatus from the white casting stand and snap into the DGGE frame with the frame lying horizontally. To snap the gel apparatus into the frame, line the top of the gel apparatus up with the white knobs on the DGGE frame and then snap the lower section of the gel apparatus into the DGGE tub frame. If only running one gel, the second gel apparatus must be must be assembled (spacers not needed) and snapped to the opposite side of the DGGE frame.

Remove ~500ml of TAE buffer from the DGGE tub. Lower the DGGE frame with attached gel apparatus into the DGGE tub. Pour the 500ml TAE buffer over the frame.

Loading Samples

Add 60ul of sample per DGGE well (or ~50µL PCR product + 15µL loading dye). Record the order by which your samples were added. Skip the two lanes on either end of the gel (can run up to 12 samples per gel).

Gently pick up and lower the DGGE top straight down onto the DGGE tub. The top is in place when the spinner is seated evenly where it belongs and you do not see the handles of the DGGE tub. Plug the electrophoresis and DGGE unit into the battery back-up unit. Attach the probes from the DGGE DCode System to the electrophoresis unit. Turn on the DGGE DCode system and check for noise, adjust the lid as necessary. Check temperature listed on the DGGE top. If temperature is $\geq$ 57°C, then turn on the electrophoresis unit. Set volts to 75V and time to 999minutes (~16.5hrs). Temperature should be set to 60°C. If the temperature is <57°C, let unit warm up before initiating electrophoresis.

Day 2 - DGGE Gel Staining and Visualization

16 hrs after the DGGE is assembled:
Note, TAE 1X is in plastic carboy near microwave, replace as needed.
You may see bubbles in glass from some gel separation
Turn off DGGE top and electrophoresis unit

Need:
- 4 containers ready, 2 for each gel + transparent transfer sheet

Add 250ml of TAE 1X and **50**-75ul (or less) of 10mg/ml EtBr to staining containers.

Pull gel(s) out of assembly, lay the frame on down and snap the plates from the frame. With the short plate facing you, place thumbs on the white spacers, slide the top of the spacers lateral to introduce air; with thumb on top of spacer and index finger on opposite side of spacer, slowly pop of the short plate. Place the transparency transfer sheet on top of the gel and flip over so that the long plate is on top and you are holding the transfer sheet in your palm; gently separate glass from the gel, trying to maintain contact between gel and transparency sheet.

Place gels in ethidium bromide (see above) for 35 minutes on agitator on speed 4; keep transparency sheet at EtBr station.

While gels are staining, rinse DGGE apparatus in DI water then soak in DI water for ~1h. Note, if running another DGGE the same day, you may reuse the TAE buffer in the DGGE tub for 1 more run.

Wash gel(s) in 300-400ml TAE 1X buffer for 20 minutes, repeat (total of 2 rinses, change out the TAE between rinses).
Using transparency sheet, transfer gel from wash tray to BioRad camera.

Open Quantity One software on PC

- Basic → Live Focus → Auto Focus w/ "Epi White" selected on BioRad camera
    - Visualize and adjust gel/zoom/focus
- Select "Trans UV" on BioRad camera → Manual Acquire, select exposure time ~7.505 (5-11+)
- Annotate and save file + export as .tiff (form used for downstream analysis)

Discard gel in EtBr solid waste bucket, discard EtBr staining solution in EtBr liquid waste, the rinse solutions and containers can be washed in the EtBr sink.

Day 2 - DGGE Gel Analysis

Resources

- Bionumerics Manual
- Shepherd #3 notebook "Hulver" p. 33, 40, 79

To transform your DGGE images to TIFF for FP Quest to understand:

- Open Quantity One (Basic) software
- Open the TDS file (Quantity One file) for the gel that you want to analyze and follow the following prompts
- File → Export → TIFF →
- Select "Export view excluding overlays"
- For the rest I use "Same as scan", "Current view", "8-bit" and "Entire image"
- I suggest retaining the same name with the new file extension so you can trace the image back to the original

To transfer your .tiff image to FP Quest for analysis

- Open the Bionumerics Manual and FP Quest Program
- Select the second button from the left "Create new database" and follow prompts on the screen (see also page 11 of the Bionumerics Manual; section 1.2.1 for database creation)
- In the FP Quest program you should see on the right hand of the screen boxes labeled "Comparisons", "Experiments" and "Files", if you do not see these, go to "Window" and select these boxes
- In the FP Quest program "key" = sample ID
- To create a fingerprint-type experiment (i.e. for DGGE), see section 2.1.3 on page 19 of the Bionumerics Manual; follow steps 3.1 through
- To import an image follow steps in 2.1.4 on page 20 of the Bionumerics Manual; do NOT edit the image upon import.
- To process the image, follow steps in 2.1.5 on page 20
    - 5.2 - alternatively, right click on the file name and select "Open fingerprint file (data)"
    - 5.3 - When the "Fingerprint type" window pops up, select the EXPERIMENT name here
    - Maximize the window to process the image and zoom out to see the whole image

        - If you see a black or white image without bands, exit and re-export the Quantity One TDS to a TIFF and re-import
    - Follow 2.1.5.1 on page 21 of the Bionumerics Manual
        - Strips tab
            - Green box - go to Lanes →Add bounding node boxes to snug the green box to the parameter of your gel
        - Curves
            - I do NOT edit tone curves
        - Normalization (Bionumerics 2.1.5.3)
            - Select a lane with conserved bands across all samples as well as across gels then select the normalization icon (this will enable you to compare samples across different gels)
            - Right click on the first band to be used as a reference position and select "Add EXTERNAL reference position"
            - Repeat for subsequent bands; I suggest picking a conserved top and bottom band
        - Bands
            - Go to Bands → Auto search bands → settings here adjusted such that they best fit banding patterns across all gels; the following settings were used for the final analysis:
                - 0% min profiling
                - 0% "Gray zone"
                - 0% min area
                - 2 shoulder sens
            - Manually select bands not auto assigned
                - Hold "Ctrl" + "Tab" while manually assigning bands
                - Can refer to the "Densitometric curve" to the right when manually assigning bands

- To link fingerprint data to entries, follow Bionumerics 2.1.6 p.28
- To run comparisons, see the Bionumerics manual

- Cluster using UPGMA and Dice's similarity matrix (Gaby 2010 and Suchodolski 2004).

DGGE Comments:

- TEMED reduces ammonium persulfate → free radical formation → vinyl polymerization of acrylamide monomers → linear chains of polyacrylamide + bifunct cross-linking agent → 3D ribbon like network of polyacrylamide
- Diameter of pores in polyacrylamide gels are determined by the concentration of acrylamide and bifunctional cross-linking agents (Chiari and Righett 1995)
    - Bifunctional cross linking agent example – N,N'-methylenebisacrylamide
- Other factors that affect band separation (Grossman 1992):
    - Gel thickness
    - Joulic heating
    - Electric field strength
- Advantages of using a polyacrylamide gel (as compared to agarose)
    - Increased resolving power; can separate molecules of DNA whos lengths differ by >0.1% (1bp in 1000bp)
    - Accommodate large quantities of DNA (i.e. $\leq$ 10µg to a single slot) w/o significant loss of resolution
    - DNA recovered is extremely pure and thus can be used for down-stream applications

Appendix M - Isolation of DGGE Bands and Cloning using the One Shot Mach1-T1 Chemically Competent Kit

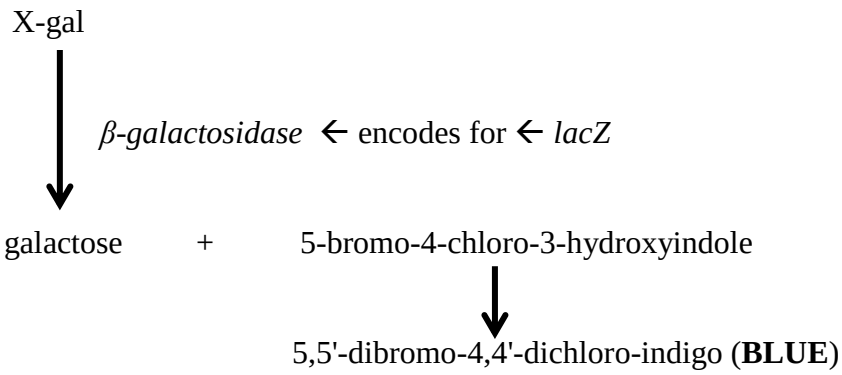
Concept

Cloning can be used as a tool to help with DGGE band identification when there is co-migration of 16S rRNA gene sequences from two different organisms within the same band. Sequences within an isolated DGGE band are amplified, inserted into a plasmid, then the plasmid is inserted into *E. coli*. The transformed *E.coli* are then cultured, harvested, and DNA is extracted (plasmid DNA including the plasmid sequence + your target sequence). One *E.coli* cell accepts only one plasmid. Therefore, if you have two bacterial 16S rRNA gene sequences in one band, one *E.coli* colony will contain the plasmid containing DNA from bact #1 and another *E.coli* colony will have the plasmid containing DNA from bact #2. Extracted DNA is then sequenced.

The Invitrogen kit simplifies what would otherwise be a lengthy process. There are two checkpoints to ensure that *E.coli* cells are transformed with a plasmid containing the unknown sequence of interest (the unknown 16S rRNA gene from the DGGE band):

1. The plasmid provided contains a kanamycin region, *E.coli* normally will not grow in the presence of kanamycin (aminoglycoside antibiotic effective against aerobic G- bacteria). However, *E.coli* harboring the plasmid will grow in the presence of kanamycin and *E.coli* that do not harbor the kanamycin plasmic will not grow.
2. The plasmid provided also contains a *lacZ* region, which encodes for the *β*-galactosidase enzyme. When the unkown 16S rRNA gene is inserted into the plasmid it is inserted between the lactose region and promoter and thus abolishes the *β*-galactosidase function. X-gal (bromo-chloro-indolyl-galactopyranoside; galactoside linked to indole), an indicator for *β*-galactosidase function, is used in the cloning media (media used to grow the transformed E.coli).

Diagram of the second check-point of cloning.



The presence of the unknown 16S rRNA gene sequence disables the *E.coli* from utilizing the lactose (your bacterial 16S DNA abolishes the *lacZ* gene). Therefore, if X-gal (along with IPTG, and an inducer of β-galactosidase) is contained within an agar medium on a culture plate, the E.coli colonies that contain the plasmid with your bacterial DNA will be WHITE. BLUE colonies represent *E.coli* colonies that contain the plasmid lacking the unknown 16S rRNA gene sequence, and thus utilized the lactose.

Cost to submit sequences to VBI (Virginia Tech, Blacksburg, VA): The cost to sequence a band ($8.50/sequence) using cloning depends on the # colonies that you harvest for sequencing. Ten colonies are typically selected from each plasmid and usually 2 plasmids are constructed per band.

Isolating DNA from DGGE Bands

Methods

Day 1

(1) Amplify DNA, per DGGE PCR protocol (Appendix L)

(2) Apply amplicons to DGGE skipping a lane between.

Day 2

(3) Stain gel per DGGE SOP. The gel was imaged prior to band excision on transiluminator. Bands were transferred to 1.5ml tubes containing 100µL RNase-free water. The UV light was turned off between band excision to prevent UV-induced DNA denaturation.

(4) Bands were incubated at 4°C overnight to allow DNA to diffuse from the acrylamide gel.

Day 3

(5) 5.4µL of Diffused DNA was amplified per DGGE PCR conditions with the exception of:

   a. The forward primer does not contain a GC clamp
      i. IDT 341F 5'-CCT ACG GGA GGC AGC AG-3' Tm58.2°C
      ii. IDT 907R 5'-CCG TCA ATT CCT TTR AGT TT-3' Tm49.6°C

(6) 25µL PCR reactions included the following

   a. 5.4µL DNA product (overnight DGGE band + RNAse free water)
   b. 1.3µL of each primer (same 907R primer + no GC-clamp 341F primer)
   c. 2µL MgCl
   d. 2.5µL DMSO
   e. 12.5µL USB Master Mix

(7) 3µL of each amplicon was checked on an agarose gel and purified for cloning.

(8) 15µL of each amplicon was run on agarose gel with your PCR products, skipping a lane between samples.

(9) Bands were excised and purified using the Zymo Gel DNA Recovery Kit (Zymo Research D4001).

(10) Purified DNA was sent to VBI for sequencing.

(11) Due to multiple sequences present within each sample, the 16S rRNA sequences within each selected DGGE band were cloned.

Cloning Isolated DNA from DGGE Bands

Materials

One Shot Mach1-T1 Chemically Competent Kit (Invitrogen #K4530)

LB Agar Kan media:

- 500ml DI water + 12.5g LB + 7.5g Agar → stir/boil → autoclave Liq3 45min → 50°C bath for 1+hr → add 2.5ml Kanamycin (10,000ug/ml) → 50°C bath until bubbles are gone → plate by pouring media into plates, pour until media covers the bottom, with lids cracked allow plates to cool → store at 4°C

Broths:

(1) LB Kanamycin Broth (w/o Agar) to use in culture tubes

- 12.5g LB broth + 500ml DI,
- Stir/boil → autoclave liq3 45min → cool on ice or room temp (no agar)
- Add 2.5ml 10,000ug Kanamycin/ml
- Store at 4°C
- 3/3/10 500ml, need to add Kanamycin

(2) LB 30% Glycerol Broth for storing cultures from broth

- 2.5g LB broth + 70ml DI water + 30ml glycerol (Fisher BP229-1)
- Stir → autoclave liq3 45min
- Store at 4°C
- 3/3/10 100ml

Culture tubes for day 2

- Add ~200µL DI water to each tube, place in rack, cover with black screw cap, autoclave liq 3 setting 45min
- Add 10ml of LB Kanamycin Broth to each tube
- Store at 4°C

Reagents:

- SOC, thaw on ice, then place in bottom 25°C incubator
- X-gal:
    - In a 1.5ml eppendorph tube, add
        - 20mg + 1ml DMSO (Fisher D128-1)
    - Dissolve by inverting, store at -20°C

Methods

Day 1

- Turn on small 42°C bath, make sure it is calibrated, check thermometer before setting temp
- Have SOC media ready, thaw on ice then place in 25°C bottom incubator
- Thaw X-gal on ice
- Thaw cloning reaction reagents: PCR product, salt solution, TOPO vector

Cloning Reactions (PCR hood, 2ml PCR tubes)

Reagent	**Chemically Competent E.coli**	**Control**
Fresh PCR product (from agarose)	2ul / 4µL	--
Salt Solution	1µL	1µL
Water	2µL / 0µL	4µL
TOPO vector (ADD LAST)	1µL	1µL
Final Volume	6µL	6µL

- Thus for each band, you will have two cloning reactions, one with 2µL of PCR product, the other with 4µL of PCR product
- As soon as the last reaction is set up, set tubes in bottom 25°C incubator EXACTLY **7min**; have **ice bucket** near the incubator
- After EXACTLY 7min, place IMMEDIATELY on ice to stop rxns
- Thaw E.coli on ice

Transformation

- Work in the hood!
- Add 2.5µL of your PCR reaction into a vial of OneShot Chemically Competent E.coli and mix by **GENTILE SWIRLING** (do not want to break E.coli cells apart)
- Incubate on **ice** for **30 MINUTES** EXACTLY (in the float)
- Incubate the cells **30 SECONDS** at 42°C small bath (heat shock for E.coli to accept plasmid w/ DNA)
- Transfer tubes to **ice** IMMEDIATELY
- Add **250µL** of 25°C **SOC** medium (promote E.coli growth)
- Cap tube, place in **37°C** incubator (top), **200rpm** shake for **1HOUR** (~2-3 E.coli generations)
- While the tube(s) is/are shaking, set up LB Kan plates, to each plate…
    - Add 40µL X-gal (20mg/ml) using the blue spreader and twirl plate
    - Parafilm plates and place upside down in 37°C incubator (top)
- For each tube (2 tubes/band) set up two plates
    - 20µL of transformed PCR product + 20µL SOC, the extra SOC is needed to allow for even spreading
    - 50µL of transformed PCR product
    - Spread onto warmed X-gal LB Kan plates, using the blue spreader and twirl plate
    - This is to ensure even spacing of colonies to make selecting colonies easier
- Incubate plates at 37°C overnight

Day 2

- Three possibilities
    - No growth = E.coli cells were not transformed, did not take up plasmic with Kan operon
    - Only blue colonies = E.coli cells were transformed, but the plasmid does not contain PCR DNA product
    - White and blue colonies = white colonies are E.coli colonies that contain the plasmid with the PCR DNA product
- Select 10 colonies/plate, for each colony…
        - Using a loop, take ½ of a colony and plate onto a grid plate, mark the plate (to ID grid orientation) and record which grid (1,2,3…) the selected colony from a specific band was placed
        - With a new loop, take the second ½ of the colony and inoculate a glass test tube containing the 10ml LB Kan broth; incubate at 37°C overnight
        - Incubate the grid plate with the broth at 37°C overnight

Day 3

- Store grid plate at 4°C fridge
- For each glass test tube…work in the hood!
    - Store one aliquot
        - Withdraw 1ml and add to a sterile 1.5ml tube
        - Centrifuge 14,000 1 min → remove supernatant
        - Add 1ml of the LB 30% Glycerol Broth → dissolve the pellet by pipetting up and down
        - Transfer to a cryovial (Corning 430658, 2ml, orange top)
        - Store at -80°C
    - Extract plasmid using the Zyppy Plasmid MiniPrep™ Kit (Zymo Research # D4019)

Appendix N - *Firmicutes* Primer Design

An attempt was made to design primers for the *Firmicutes* phyla members using sequences obtained from the pyrosequencing data in Chapter 5. Sequences from 8 selected genera representing the *Firmicutes* phyla (Table 30) were aligned using Genious software (www.geneious.com) (Figure 11). Aligned sequences were evaluated for two regions (19-23bp in length) conserved among *Firmicutes* sequences, but not non-*Firmicutes* sequences designed to produce amplicons 50-210bp in length. Unfortunately, due to the relatively short sequence lengths (250bp), we were unable to find conserved regions of aligned *Firmicutes* members. Full sequencing of the target gene, such as 16S, or whole genome from *Firmicutes* members isolated from the horse is needed in order to have an appropriate template to design primers, which would involve target bacterial isolation and sequencing of the complete 16S rRNA genome.

Methods

Sequences were selected from the pyrosequencing data described in Section 4. The *Firmicutes* and non-*Firmicutes* sequences used to design primers are listed in Table 30 below.

Table 30 - *Firmicutes* and non-*Firmicutes* sequences used for primer design.

Phyla	Genera	Contiguous sequence ID
Firmicutes	*Mogibacterium*	Contig_2693N42
Firmicutes	*Blautia*	Contig_5066N4
Firmicutes	*Eubacterium*	Contig_4975N4
Firmicutes	*Acidaminococcus*	Contig_4756N4
Firmicutes	*Ruminococcus*	Contig4681N2
Firmicutes	*Roseburia*	Contig_4996N4
Firmicutes	*Streptococcus*	Contig_1985N3
Firmicutes	*Lactobacillus*	Contig_663N3
Proteobacteria	*Desulfovibrio*	Contig5103N95
Verrucomicrobia	*Akkermansia*	Contig5993N173
TM7	Incertae_sedis	Contig4609N69
Bacteroidetes	*Prevotella*	Contig1980N9
Actinobacteria	*Denitrobacterium*	Contig467N90

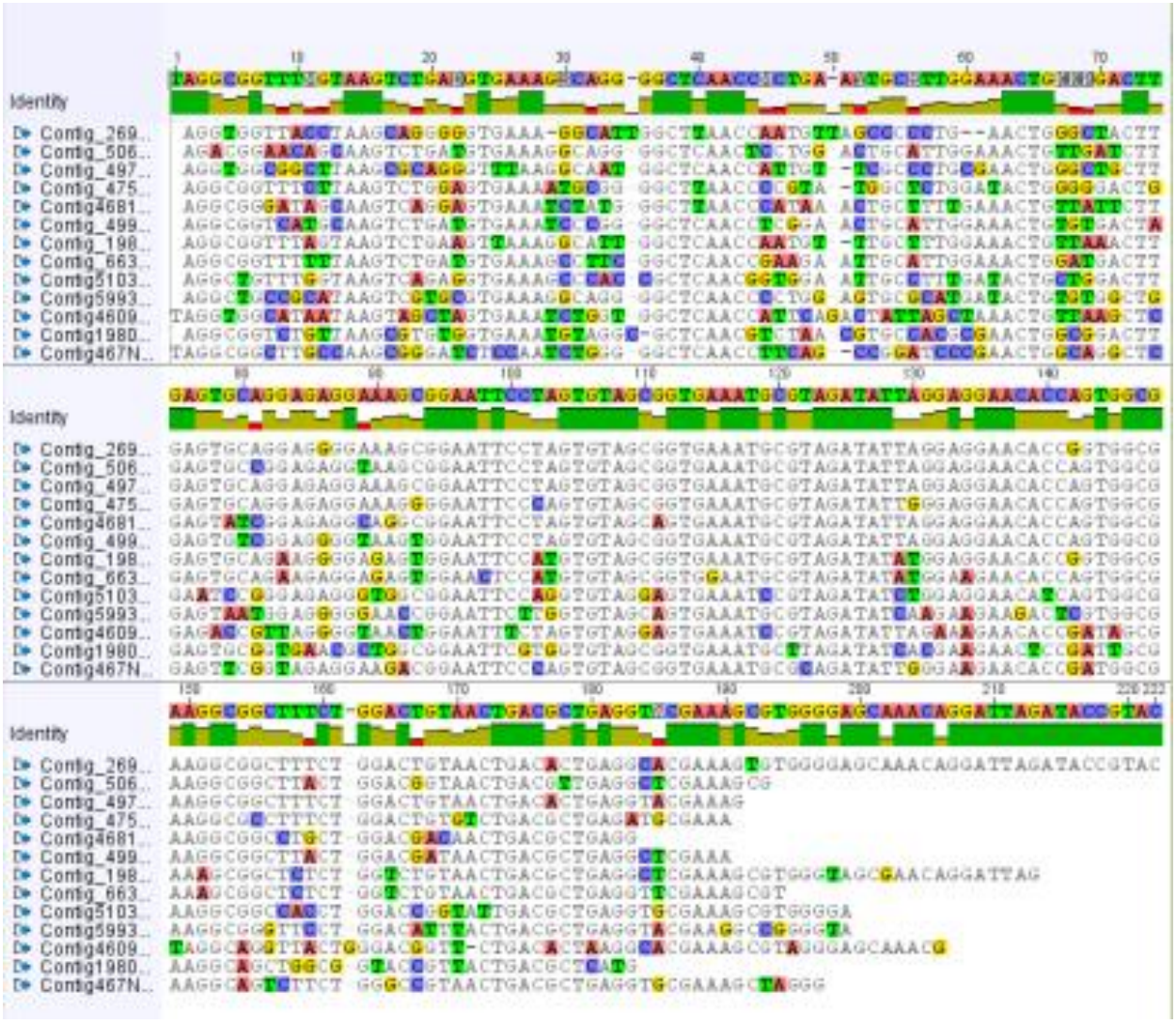
The sequence identification (i.e. Contig_1985N3) was an identidy assignment by Lasergene (DNAStar, Madison, WI), a software that Dr. Jensen used for processing the raw pyrosequencing data (Section 5; sequences were deposited in the GenBank Sequence Read Archive [http://www.ncbi.nlm.nih.gov/genbank/ and can be accessed by searching for accession # SRA039855 at http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj]); identity assignment is based on abundance. For example, Contig_2693N42 means that the contiguous sequence identified as 2693 is present 42 times. Therefore, the sequence corresponding to the genera Mogibacterium is present 42 times in the sequences constructed from all 4 fecal samples.

Sequences were aligned using Geneious software (Figure 11) and subjectively inspected for two 19-23bp (Quellhorst and Rulli, 2008) conserved regions present only in the *Firmicutes* sequences to which *Firmicutes* primers could be designed.

Discussion

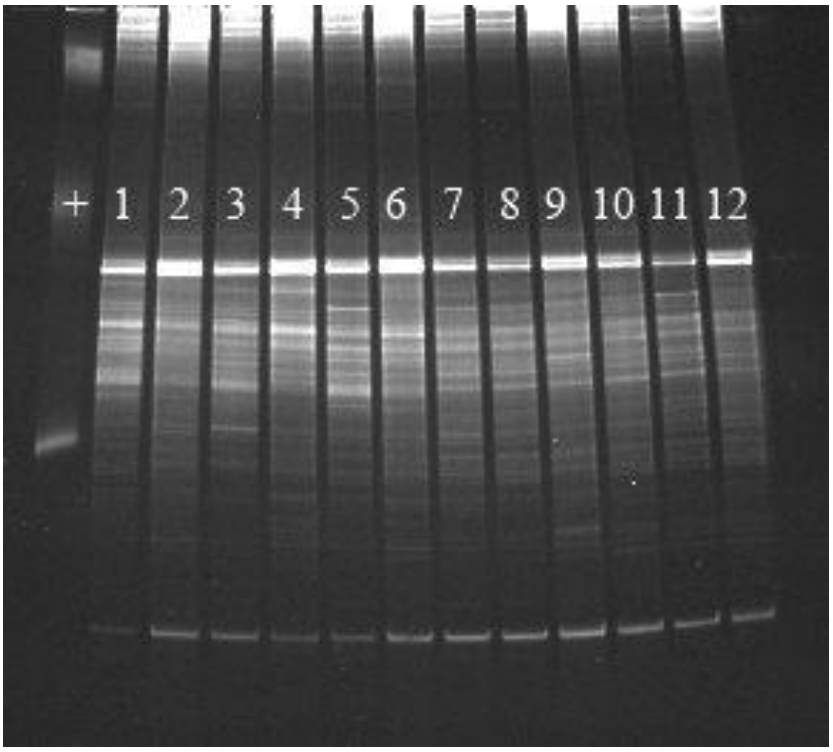
Conserved regions were not identified from the selected *Firmicutes* sequences. The De Greoris *et al* (2011) primers were selected to evaluate the abundance of total bacteria and *Firmicutes* members in Section 6 based on real-time PCR results as described in section 4.4.5. DeGregoris *et al.* (2011) designed primers (Table 7) based on 16S rRNA gene sequences uploaded to the RDP database. For each primer set, 20-30 16SrRNA gene sequences were aligned for primer design. Primers were validated by use of end-point PCR with target and non-target DNA and sequenced clone libraries generated from end-point PCR products. Furthermore, the primers were modified to use the same thermocycle protocol to allow for applying multiple primer sets to each plate to eliminate the effect of intra-plate variation in results. DeGregoris *et al.* (2011) reported that the proportion of RDP Probe Match hits and portion of target bacterial taxa that complement the primer to be 96.7% and 95.3%, respectively, for the cfb967R primer and 99.4% 83.2%, respectively, for the 1040firmR primer. Furthermore, *Firmicutes* primer specificity, as determined by product sequencing, was 56% for the 928firm + 1040firmR primer sets and 100% for the LGC353+EUB518 primer set.

Figure 11 - ***Geneious® (www.geneious.com) alignment of the eight Firmicutes sequences and 5 non-Firmicutes sequences (*****Table 30*****) for the purpose of Firmicutes primer design.***



Appendix O - DGGE Gels

Figure 12 - ***DGGE gel of fecal bacteria[a] from Arabian geldings fed an18% or 12% NSC hay.***

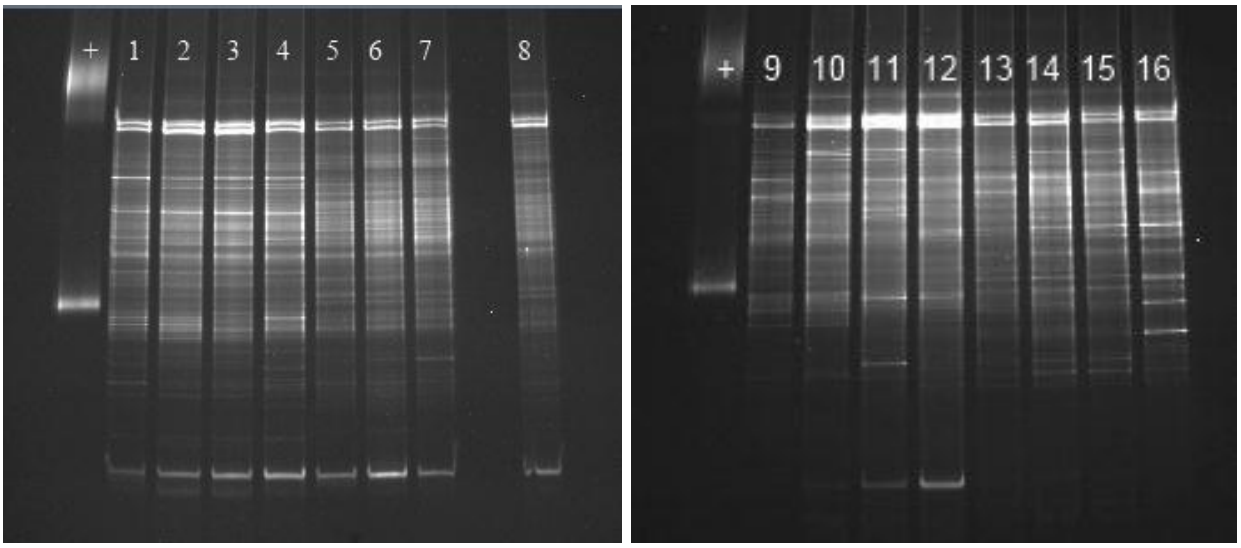


Columns are identified by number and correspond to the table below.

Lane	Horse ID	Date of Fecal Collection	Time of Day of Fecal Collection	Hay
+	Positive control			
1	244	12/15/08	AM	18% NSC
2			PM	
3		12/16/08	AM	
4			PM	
5		12/17/08	AM	
6			PM	
7	248	12/15/08	AM	12% NSC
8			PM	
9		12/17/08	AM	
10			PM	
11		12/18/08	AM	
12			PM	

[a]*DNA was extracted using the MoBio Ultra Clean® Fecal Kit.*

Figure 13 - ***DGGE gels representing the banding pattern of fecal bacteria, from four Arabian geldings fed an18% or 12% NSC hay.***

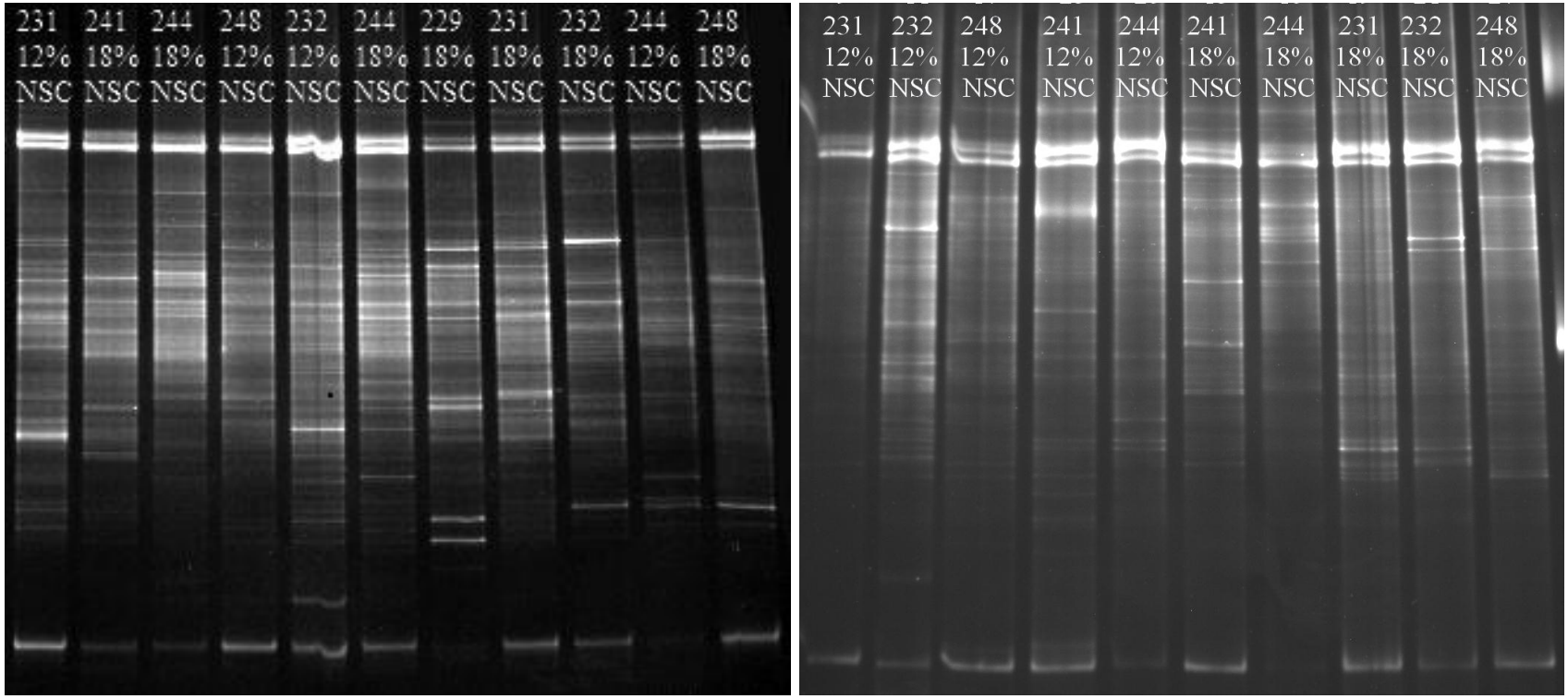


Columns are identified by number and correspond to the table below.

Lane	**Horse ID**	**Date of Fecal Collection**	**Hay**
+	Positive control		
1	232	1/19/09	18% NSC
2		1/20/09	
3		1/21/09	
4		1/22/09	
5	244	1/19/09	12% NSC
6		1/20/09	
7		1/21/09	
8		1/22/09	
9	248	1/19/09	18% NSC
10		1/20/09	
11		1/21/09	
12		1/22/09	
13	249	1/19/09	12% NSC
14		1/20/09	
15		1/21/09	
16		1/22/09	

Figure 14 - ***DGGE gels of fecal bacteria extracted using the MoBio Ultra Clean® Fecal Kit (A) and the Zymo Soil Microbe DNA Kit (B), from Arabian geldings fed an18% or 12% NSC hay.***

A **B**



Columns are identified by horse ID and hay type.

Appendix P - SAS Code for the GLIMMIX Procedure for Section 4 qPCR

Data Analysis

```
dm'clear log'; dm'clearoutput';
PROC IMPORT OUT= VHIB06302012b
DATAFILE= "P:\Private\BMVS PhD\VHIB 2009\qPCR\SAS\VHIB SAS qPCR.xls"
DBMS=XLS REPLACE; SHEET="SAS 231 244 248 noWO"; GETNAMES=YES; MIXED=NO;
     /*SCANTEXT=YES; USEDATE=YES; SCANTIME=YES;*/

options helpbrowser=sas;
ods graphics on;
ods rtf;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model totalbacteria_F338_R518 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model totalbacteria_F926_R1062 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model firmicutes_FLGC353_R518 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
```

```
  model firmicutes_F934_R1060 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model firmicutes_F928_R1040 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model FLGC353_R518_F338_R518 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model firmicutes_F934_R1060_F338_R518 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=VHIB06302012b plots=studentpanel;
  class hay horse_ID;
  model firmicutes_F928_R1040_F926_R1062 = hay;
  random horse_ID(hay);
  lsmeans hay;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;
```

```
data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

ods rtf close;
ods graphics off;
```

Appendices to Section 5

Appendix Q - Pyrosequencing Concept

In 2010 there were three platforms by which nucleic acids could be sequenced, AB Solid, 454 FLX, and Solexa. These platforms provide newer technology than the Sanger technology. Of the three available sequencing platforms, the Roche 454 FLX technology offers a compromise between longer read lengths (250-500bp), as compared to Solexa (up to 150bp), and number of reads per run (Parameswaran et al., 2007).

The pyrosequencing process was originally described by Ronaghi *et al.* (1996; 1998) and Nyren *et al.* (1994; 1997). After extraction, DNA is amplified using primers with unique linker sequences. The linker sequences attach to amplicons and anchor the amplicons to the pyrosequencing bead. Amplicons are then distributed onto a PicoTiter Plate™ containing thousands of 29µm wells, each with a single pyrosequencing bead (Roche FLX; http://www.454.com/). Each well is subsequently occupied by an individual unknown sequence. The amplified DNA incubated with DNA polymerase, adenosine 5' phosphosulfate, sulfurylase, luciferase and apyrase. Each deoxyribonucleotide triphosphate (dNTP) is added one at a time (i.e. A is added to all wells first). If the complimentary base is present (i.e. T) in the unknown sequence, DNA polymerase adds the dATPs to the 3' phosphate end of the synthesis strand (strand opposite to the unknown strand). A pyrophosphate is formed with each dNTP that is added and sulfurylase then catalyzes the conversion of pyrophosphate + adenosine 5' phosphosulfate to ATP. ATP-driven luciferase then catalyzes the conversion of luciferin to oxyluciferin, which ultimately produces light. The light is then detected by a charge coupled device camera, which then produces a peak in the Pyrogram (Ahmadian et al., 2006). The peak height is positively correlated with number of nucleotides that complement the next base of the unknown sequence. Apyrase then degrades ATP and remaining dNTPs prior to the addition of the next dNTP (i.e GTP) (Ahmadian et al., 2006). This process continues with each base and repeats until the complimentary DNA strand is complete. The Pyrogram output then reads as a sequence of light peaks associated with each nucleotide added. The output is then trimmed of linker sequences. The remaining contiguous sequences, set of overlapping DNA sequences, are then trimmed and compared to a database such as the Ribosomal Database Project (Cole et al., 2005).

Appendix R - Expansion of Materials and Methods from Section 5

Generation of PCR Amplicons

Amplicons were generated using primers described in Table 31. The PCR reaction consisted of 25 µL HotStart-IT™ FideliTaq™ PCR Master Mix (2X) (71156, USB), 3.5 µL of each primer (10 µM), 2 µL $MgCl_2$ (24mM), 5 µL DMSO (10%), 100ng DNA, and RNAse-free water to make a 50 µL total volume. The PCR protocol consisted of denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, annealing at 60°C for 45 sec, and elongation at 72°C for 1 min, and a final elongation at 72°C for 7 min.

Table 31 - ***Primers used in this study were designed by Michigan State University (http://pyro.cme.msu.edu/) (Cole et al., 2009).***

Primer	Sequence (5' → 3')
Forward [a]	**GCCTCCCTCGCGCCATCAG**AYTGGGYDTAAAGNG
Reverse 1[b]	**GCC TTG CCA GCC CGC TCA G**TA CCR GGG THT CTA ATC C
Reverse 2[b]	**GCC TTG CCA GCC CGC TCA GT**A CNV GGG TAT CTA ATC C

Bolded sequence corresponds to the 454 Life Sciences primer A[a] or primer B[b] (Roche), the remaining sequence corresponds to the V4 region of the bacterial 16S rRNA sequence.

Processing Amplicons for Pyrosequencing

Amplicons were run on a 1.5% agarose gel and bands were isolated and purified using the QIAquick PCR Purification Kit (28106, Qiagen). Purified DNA was quantified by spectroscopy (Nanodrop) and standardized to a 35 ng/µL concentration. Products from the two reverse primer were combined within sample.

Samples were sequenced using the 454 GS FLX system by Roche at the Virginia Bioinformatics Institute (VBI, Blacksburg, VA). Sequencing was performed off the forward primer linker (A) sequence. Dr. Rick Jensen processed raw sequence results, which included: trimming linker sequences, removal of sequences <200bp, removal of sequences with a single occurrence (represented by a single read), and construction of contiguous sequences. To trim linker sequences, Dr. Jensen manually removed 20 bases of the 5' end and 30 basis of the 3' end.

A contiguous sequence is an assembly of overlapping DNA fragments; the DNA fragments represent a continuous region of DNA. Pyrosequencing identifies the nucleotide sequence of 250-260bp fragments. Any two or more sequences could represent the same gene segment. Contiguous sequences in the present data were constructed using Lasergene (DNAStar, Madison, WI); sequences were considered contiguous if there was 100% match over 25 bases. Contiguous sequences (contigs) were assigned an identity based on abundance such that contig 3519N391 means that there are 391 reads that match the sequence of contig 3519; therefore the number after N represents the abundance of contig 3519.

Uploading Sequences to the RDP Database

Materials

1. Google chrome
2. FASTA file of sequences to be analyzed; the data must be trimmed
    a. This was performed by Dr. Jensen

Methods

1. Go to the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/)
2. Select Pyrosequencing Pipline (http://pyro.cme.msu.edu/)
    a. Note, words in **bold** below correlate with the links in green on the left column of the RDP Pyrosequencing Pipeline website
3. Go to **Aligner**
    a. Upload FASTA file (one file at a time) → RDP will send an e-mail with a link, select the link → save as → right click → open containing folder → copy .tgz to correct folder
        i. .tgz is a compressed file, this contains a .FASTA file, .txt, and an image file; you want the .FASTA for clustering
4. Go to **Complete Linkage Cluster**
    a. Upload .FASTA file from **Aligner**
    b. Will get an e-mail with a .tgz, which contains a .clust + other files; you want the .clust file
    c. Create SEPARATE cluster/sample for each sample for **Shannon and Chao1 Index** and **Rarefaction**
        i. Distance = % dissimilarity among sequences; use 3% dissimilarity
    d. Create ONE cluster with all samples to use for **Jaccard and Sorensen Index**
        i. Will get another e-mail w/ a cluster.tgz file, again, want the .clust file
5. Go to **Shannon and Chao1 Index** (from separate cluster files, 1 cluster/sample)
    a. Import cluster tgz file for each sample separately
    b. Chao = richness estimator, # of sequences or OTUs that fit the cluster
        i. LCI, UCI upper and lower limit for richness

c. H = Shannon index = diversity, # organisms/cluster, compare individual samples to show which sample has more/less diversity

$$H = \sum_{i=1}^{s} - (P_i * \ln P_i)$$

i. Calculated as where:

1. H is the Shannon diversity index
2. P_i is the fraction of the entire population made up of $_i$ species
    a. P_i = # of individuals in species #1 / total # of individuals of all species
    b. Repeat this for each species present; through species "S" (the last species encountered)
3. S is the numbers of species encountered
4. $\sum$ is the sum of species 1 to species S

ii. The value for H is positively correlated with community diversity

1. H = 0 represents a community with only one species

d. Save .txt file, open in excel

i. Open excel then select open → all files → select the txt file from Shannon Cho

ii. Diversity = richness + abundance

iii. Richness = # of diff organisms in a sample

1. 0.03 = 3% dissimilarity = 97% alignment

6. Go to **Rarefaction**

a. Import cluster file

b. Save .txt file, open in excel and save

i. 1st column = # sequences, other columns for % dissimilarity, further right are columns with lower and upper limits for various % dissimilarity

ii. Can make a new sheet with the # of sequences (1st column) and the column with 0.03% dissimilarity

iii. Plot x = # seq (1st column) by y = % dissimilarity column (i.e. 0.03)

1. Can plot all 4 samples once you have performed individual rarefraction analysis on them IF using the same % dissimilarity OR

plot each sample on a separate graph using more than one % dissimilarity

7. Go to **Jaccard and Sorensen Index** - Will make dendogram to compare all 4 samples
    a. Use files that have been individually aligned (each sample file aligned individually)
    b. Create a single cluster file with the 4 samples
        i. Import allsamplesmegan.clust
    c. Ask for both Jaccard and Sorensen Index
        i. Will have a .tgz file automatically downloaded from the website (not e-mailed)
        ii. There will be lots of files; you want the one representing the tree representing 0.03 dissimilarity
        iii. UPGMA (unweighted pair group method with arithmetic mean) = mathematical model to make the cluster for both Jaccard and Sorensen; use Jaccard
8. Go **RDP Classifier** – For determining the abundance of each contiguous sequence
    a. Upload .fasta files
    b. To present relative abundance, use info to make tables with each sample = 1column, Y axis rep the % abundance of each group of bacteria
9. Finally, sequences must be uploaded to NCBI prior to publication.
    a. Will need an .ftp file to upload sequences to; request this through NCBI

Appendix S – Section 5 Pyrosequencing Results from Individual Samples

Table 32 - *Richness and diversity estimators that predict the number of unique sequences in the forage-fed horse's fecal microbiome.*

Factor	Non-Obese 12% NSC	Non-Obese 18% NSC	Obese 12% NSC	Obese 18% NSC
Chao1 Index (S)[a]	2,157	2,154	3,163	1,963
Shannon Index (H')[a]	6.6	6.5	6.9	6.6
Evenness (E)[b]	0.86	0.85	0.86	0.87

[a]Chao1 and Shannon Index calculated at 3% dissimilarity; [b]Evenness was calculated as E=H'/Hmax; Hmax = ln(Chao1)

Figure 15 - *Relative abundance of the four dominant phyla in the four fecal samples.*

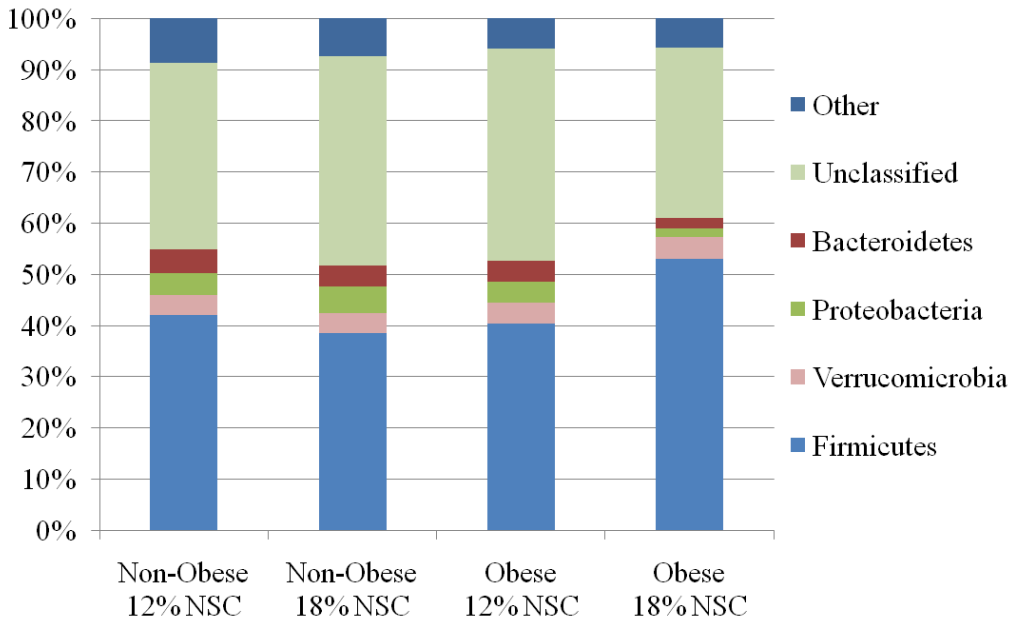


Figure 16 - ***Rarefaction curves, calculated at 3% dissimilarity, representing OTUs within the fecal microbiome in the four fecal samples.***

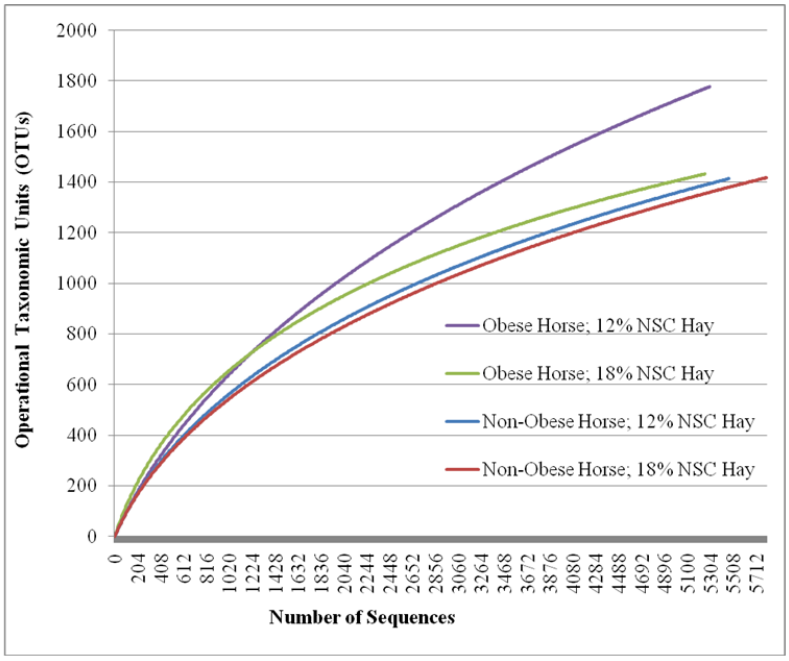
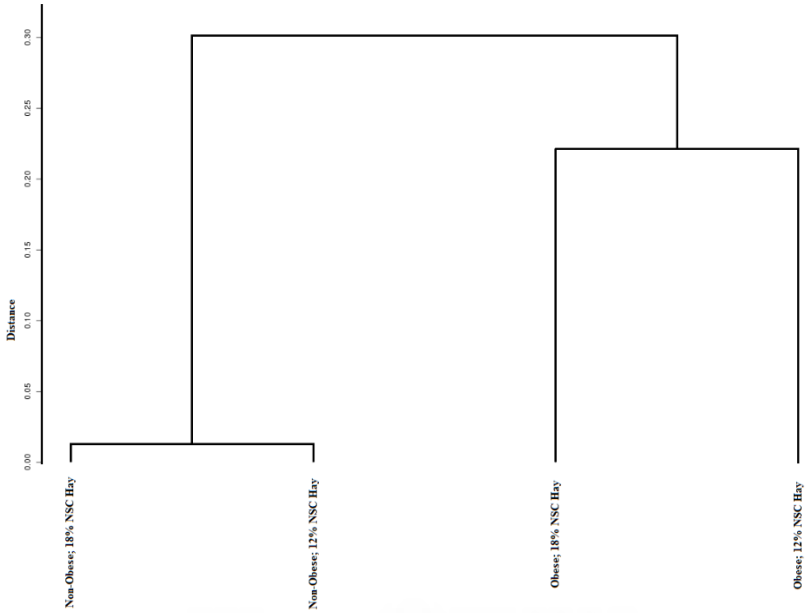


Figure 17 - ***Dendrogram representing fecal microbiome similarity between the 4 gelding samples.***



Appendix T – Expansion of Discussion from Section 5

Preliminary Evaluation of the Effect of High vs. Low NSC Hay on the Fecal Microbiome of Arabian Geldings

The bacterial richness (Chao1 index) of the obese gelding increased by 400 OTUs when fed the moderate (12% NSC) NSC hay as compared to the other three samples. The abundance of *Fibrobacter* spp., in the feces of horses consuming the low NSC hay was numerically higher (0.8% of total sequences) than in the feces of horses consuming the high NSC hay (0.7%). This is not surprising as the moderate NSC hay was higher in fiber (70% NDF) than the high NSC hay (61% NDF). The abundance of *Streptococcus* spp. in the present study was numerically higher (0.1% total sequences) in geldings fed the moderate (12%) NSC hay as compared to geldings fed the high (18%) NSC hay (0.2%). Fructan utilizers were represented by *Treponema* spp. (1.9% of total bacteria), a member of the *Spirochaetes* phylum (Piknova et al., 2008). *Cyanobacteria* have been identified in the gut of mice and humans (De Gregoris et al., 2011; Eckburg et al., 2005; Ley et al., 2005; Stearns et al., 2011). The abundance of unclassified sequences were higher in the present study than reported in human studies (Stearns et al., 2011).

Treponema spp. represented 2.5% and 1.4% of total fecal bacteria when horses were fed the moderate (12%) and high (18%) NSC hay, respectively. *Treponema* spp. is a hydrogen utilizing acetogen that represents a small proportion (3%) of equine hindgut microbes (Daly et al., 2001); although, the relative abundance is higher than reported in cattle feces (0.93%) (Dowd et al., 2008). Methane production in the horse is less than that of ruminants (Vermorel, 1997), which may be due to the higher abundance of *Treponema* spp. competing for hydrogen. The abundance of these *Treponema* spp. and methanogens is inversely related in the termite gut and human oral cavity (Leadbetter et al., 1999; Lepp et al., 2004).

The data presented here serves as preliminary data as an effect of diet on the fecal microbiome of Arabian geldings could not be evaluated due to the limited number of fecal samples evaluated (n=4). Costa and Weese (2012) contrasted this work with their findings presented as a conference poster (Costa et al., 2011) (Table 33) were both authors evaluated fecal bacterial communities by use of 16S rRNA pyrosequencing. The present author targeted the V4 region of the 16S rRNA gene of equine fecal bacteria in health horses; Costa *et al.* (2011) targeted the V3-V5 16S rRNA gene region of fecal bacteria in normal vs. diarrheic horses.

Table 33 – ***Comparison of the fecal bacterial communities, expressed as relative phyla abundance, using high-throughput pyrosequencing between two studies.***

	Shepherd *et al*. (2012b)	**Costa *et al*. (2011)**
Targeted 16S rRNA gene region	V4	V3-V5
Roche 454 Pyrosequencing Platform	GS FLX	GS Junior
Ave. # sequences/sample	28,458	12,901
Ave. sequence read length	250	523
16S rRNA gene database	RDP (rdp.cme.msu.edu)	SILVA Small Subunit rRNA database
	Phyla (% total bacteria)	
Firmicutes	43.7	65.0
Verrucomicrobia	4.1	Not reported
Proteobacteria	3.8	12.0
Bacteroidetes	3.7	14.0
Spirochaetes	2.1	3.4
TM7	1.8	Not reported
Actinobacteria	1.6	4.0
Fibrobacteres	0.8	Not reported
Tenericutes	0.1	<1%
Cyanobacteria	0.1	<1%
Fusobacteria	Not identified	<1%
Synergistetes	Not identified	<1%
Unclassified bacteria	38.1	0.5

The sequence read length reported by Costa *et al.* (2011) is high relative to the read length that Roche publishes (~400bp) (http://454.com/products/gs-junior-system/index.asp); their primers provided a longer amplicon that in the present study, which improves sequence identity, allowing for deeper taxonomic classification. The present author did not utilize the full potential of the FLX pyrosequencing technology as the average read length presented here was lower (ave 250bp) compared to what Roche publishes for their FLX Titanium XLR70 platform (http://454.com/products/gs-flx-system/index.asp). Costa *et al.* (2011) reported a lower proportion of *Firmicutes* and a higher abundance of *Proteobacteria, Bacteroidetes, Spirochaetes,* and *Actinobacteria*. However, there was large variation in relative bacterial phyla abundance between the 6 normal horses in the Costa *et al.* (2011) study; for example, the relative abundance of *Firmicutes* members ranged from 28% to 71%. The lower abundance of unclassified bacteria, as reported by Costa *et al.* (2011), is likely due to the longer read lengths (523bp) as compared to the present work (250bp) and is likely the cause for the difference in the relative abundance in bacterial phyla. The difference in the 16S rRNA gene region targeted by Shepherd *et al.* (2012b) vs. Costa *et al.* (2011) may not have influenced the phyla-level differences between these two studies as Kumar *et al.* (2011) did not report a difference in bacterial diversity or number of phyla in human subgingival plaque samples when targeting the V1-V3 vs. V4-V6 16S rRNA region.

Limitations

The primers used in the present study produced a 250bp product and therefore, did not maximize the 250bp limit of 454 Life Sciences™ technology (http://www.454.com/). The primers used in the present study had been previously used in the lab to evaluate the microbial communities of spinach (Lopez-Velasco et al., 2011) and other primer options were not considered. Should future high-throughput sequencing be used in the future, the authors would consider two options:

1) Identification or primers that would produce an amplicon size to maximize the capacity of the sequencing technology used.

2) Sequencing directly from extracted DNA without amplification. The benefit of DNA amplification and purification is to produce a more pure DNA product (i.e. DNA fragments are eliminated) including amplicons of a target gene and of similar length. However, amplification prior to sequencing introduces PCR bias. Therefore, sequencing DNA without

amplification would provide a more accurate measurement of abundance. The PCR step prior to pyrosequencing is necessary due to the need for linker sequences to anchor the bacterial sequence to the pyrosequencing bead.

Although pyrosequencing is sensitive tool to identify members of a complex bacterial community (Trochimchuk et al., 2003), it is not a preferred tool for the evaluation of microbial abundance. The limitations of pyrosequencing include:

1. **PCR Bias** as previously described by the present (Section 5.4) and previous authors (Juste et al., 2008; Kanagawa, 2003). The bias of PCR can be reduced by limiting the number of thermocycles.
2. **Short Sequence Read Length** (250-300bp) - Limitations in sequence read length are due to the pyrosequencing bead size. Short read lengths limits taxonomic specificity.
3. **Chimeric sequences** – hybrid of two parent sequences formed during PCR.
4. **Sequencing errors** (e.g. mismatch, insertion/deletion)
5. **Computational Bias** - Contiguous sequence assembly may be performed with a one of several databases including Lasergene Megaline (http://www.dnastar.com/) (DNAStar, Madison, WI), MeganDB (http://www.megan-db.org/megan-db/), NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), or RDP (rdp.cme.msu.edu) (Cole et al., 2005). There is no gold standard and computational databases are constantly evolving. Comparison of results across studies can be challenging because there is no data analysis standard and results from the different databases will differ depending on the sequences deposited in a given database.

New Technology

High through-put sequencing technology is constantly evolving and changes with newer technology include both lower cost and increased sequence read length. New sequencing platforms include PACBio RS (www.pacificbiosciences.com) and Ion Torrent Proton (www.iontorrent.com). Not all high-throughput sequencing platforms are limited to specialized labs as there are bench-top technologies available including the 454 GS Junior (Roche), Ion Torrent Persona Genome Machine (PGM) (www.iontorrent.com; Life Technologies), and MISeq (Illumina). The bench-top version of the 454 technology, GS Junior, provides a mean read length of 522bp, which is the longest of the bench-top sequencers (Loman et al., 2012). Ion Torrent technology works much like pyrosequencing technology (i.e. similar sequencing methodology and read length); however, in place of light, the instrument detects hydrogen ions produced each time a nucleotide is incorporated into the synthesis strand.

High throughput sequencing technology can be used for identification of DNA, RNA (cDNA), and protein structure. Therefore, sequencing technology is not limited to genomics, but may be applied to transcriptomic and proteomic work to be used for evaluation of functional capacity.

Appendices to Section 6

Appendix U - Ankom Fiber Analysis Protocol for Determination of Neutral Detergent Fiber (NDF)

Concept

During NDF analysis, ground forage samples are digested in fiber bags using a detergent solution. Forage molecules such as soluble carbohydrates (mono/disaccharides, starch, fructan, and pectin), protein, and cell wall lipids are washed away (out of the fiber bag) leaving behind cellulose, hemicellulose, and lignin.

Materials

1. Neutral-detergent solution (NDS)
    a. 18.0 L Deionized Water
    b. 1200.0g Neutral Detergent Dry Concentrate (Ankom # FND20C)
    c. 200.0 ml Triethylene glycol
2. Sodium sulfite (Na2SO3)
3. Heat-stable alpha amylase (activity = 340,000 Modified Wohlgemuth Units/ml) – used for starch digestion
4. Acetone (CH3COCH3)

Methods

1. Add 2000 ml of ambient NDS into a digestion vessel (Ankom 200 Fiber Analyzer Model A200, Ankom Technology, Macedon, NY)
2. Place plastic bag suspender with bagged samples into the solution in the digestion vessel
3. Turn *agitate* and *heat* on and close the lid and seal the digestion vessel
4. After 60 min have elapsed turn *agitate* and *heat* off and open the valve slowly and exhaust hot solution before opening the lid
5. After the solution has exhausted, close the valve and add 2000 ml of 90 – 100°C H2O and 4 ml alpha-amylase
6. Lower the lid to the digestion vessel but do not tighten.

7. Turn *agitate* on and leave the *heat* off for 5 minutes
8. Exhaust liquid in digestion vessel and then close valve
9. Repeat Steps 5 – 8
10. Repeat steps 5 – 8 without adding alpha-amylase
11. Remove filter bags from bag suspender and gently press out excess water
12. Place filter bags in beaker and soak in acetone for 3 minutes
13. Remove filter bags from acetone bath and lightly press out excess acetone
14. Spread bags out and allow acetone to evaporate completely in fume hood
15. Complete drying in an oven at 105°C for at least 4 hours
16. Remove bags from oven and place directly into sealable plastic bag containing a desiccant and flatten to remove air
17. Cool bags to ambient temperature and weigh filter bags with samples
18. Record weight

Appendix V - Ankom Fiber Analysis Protocol for Determination of Acid Detergent Fiber (ADF)

Concept

During ADF analysis, dried forage bags used for NDF analysis are further digested in an acid (sulfuric acid) and detergent solution. Forage hemicellulose is washed away from the fiber bag leaving behind cellulose and lignin.

Materials

1. Acid detergent solution (ADS)
2. 200g Hexadecyltrimethylammonium bromide ($CH_3(CH_2)15N(CH_3)3Br$)
3. 10 L 1 N sulfuric acid (H_2SO_4)
4. Acetone (CH_3COCH_3)

Method

1. Add 2000 ml ADS into digestion vessel
2. Place plastic bag suspender with bagged samples into the solution in the digestion vessel
3. Turn *agitate* and *heat* on and close the lid and seal the digestion vessel
4. After 60 min have elapsed turn *agitate* and *heat* off and open the valve slowly and exhaust hot solution before opening the lid
5. After the solution has exhausted, close the valve and add 2000 ml of 90 – 100°C H2O
6. Turn *agitate* on and leave the *heat* off for 5 minutes
7. Exhaust liquid in digestion vessel and then close valve
8. Repeat Steps 4 – 7 twice
9. Place filter bags in beaker and soak in acetone for 3 minutes
10. Remove filter bags from acetone bath and lightly press out excess acetone
11. Spread bags out and allow acetone to evaporate completely in fume hood
12. Complete drying in an oven at 105°C for at least 4 hours
13. Remove bags from oven and place directly into sealable plastic bag containing a desiccant and flatten to remove air
14. Cool bags to ambient temperature and weigh filter bags with samples

15. Record ADF bag weight
16. Subtract ADF bag weight from the bag weight after 0.5 g 1mm ground sample had been added in the preparation step
17. Divide value determined in step 16 by 0.5 then multiply that value by 100 to determine percent ADF of sample placed in filter bag
18. Divide value determined in step 17 by the average micro dry matter weight of the forage sample to determine the percent of ADF in the dry matter of the forage sample

Appendix W - Preparation of Equine Feces for Derivatization

Concept:

The purpose of preparing equine fecal samples for derivatization is to preserve volatile fatty acids in their polar (anion/ionized) form.

Materials:

- Ethanol
- Spatula
- Scale
- 50ml tube rack
- 50ml tubes (1/sample)
- Fisherbrand 1DR glass screw-top vials (1/sample) (Fisher 03-0338A; 15x45mm)
- 3ml syringe w/ attached needle (from VTH stores)
- Fisher transfer pipets (1) (Fisher 13-711-9AM)
- Serological pipette 10ml (1)
- Syringe filters - 0.20uM (1/sample) (Corning 431215, RC Inc., Corning, NY)

Method:

(Otto ER *et al*. 2003. Ammonia, volatile fatty acids, phenolics, and odor offensiveness in manure from growing pits fed diets reduced in protein concentration, J Anim Sci 81:1754–1763.)

1. Before starting:
    a. Pull samples to thaw
    b. Turn on centrifuge, set to 4°C
    c. Label the 50ml tubes and glass screw-top vials (use tape)
2. In a 50ml centrifuge tube, add
    a. 2g thawed sample
    b. 8 mL of DI water
    c. Two drops of concentrated HCl (using a transfter pipet)
3. Vortex max speed 20 sec
4. Centrifuge at 25,314 × *g* for 10 min at 4°C

5. Collect the supernatant using a 3ml syringe and needle
6. Attach a 0.20-µm filter to the syringe and filter the supernatant into a Fisherbrand 1DR glass screw-top vial
7. Store at -20°C until derivatization (see Appendix D.4)

Appendix X - Derivatization of Prepared Equine Feces and Plasma for VFA Analysis

Modified 10/18/11 by Megan Shepherd from the 1/20/10 version by Tara Wiles (originally adapted from Kristensen 2000 ACAS 50)

Concept:

Volatile fatty acid derivatization is a process of producing VFA derivatives through esterification to reduce VFA polarity (as compared to their free acid form) and to subsequently reduce sample carry-over (Husek et al., 1990; Kristensen, 2000). Free fatty acids (i.e. VFAs) may be applied to gas chromatography (GC) in free form; however, these polar acids can bind to the column reducing the molecules reaching the mass spectrometer (MS) and resulting in sample carry-over. Therefore, the purpose of derivatization is to esterify the fatty acids to both increase sensitivity of GC-MS and reduce sample carry over in the GC column. During derivatization in the present protocol, free VFAs are esterified using chloroformate. The presence of ethanol improves VFA esterification with chloroformate. Acetonitrile is an organic solvent with high polarity and this is a desirable trait when esterifying water-soluble molecules (i.e. VFAs). Pyridin functions as a catalyst and acetonitrile serves as a reaction medium. Heptan is a solvent that improves the retention time of esterified VFAs in the GC column.

Materials

- Screw cap culture vials (100 x 16 mm) - preferred sample storage
- Centrifuge - frigerated
- Autosampler vials with inserts and caps
- Nitrile gloves
- Three separate waste bottles (in Hanigan hood)
- Sharpie
- Lab coat

Chemicals

- 1 mM [1-C13, D3]Sodium **acetate** (Cambridge Isotope Labs CDLM-611-1)

- 1 mM [2,2-D2]**Propionic acid** (Cambridge Isotope Labs DLM-2488-1)
- 1mM [1,2 -$^{13}C_2$] Sodium **butyrate** (Sigma, Isotec 491993-100MG)
- 2-Chloroethanol *highly TOXIC* (Fluka 23000) - in tin in hood
- Acetonitril (ACN) - in yellow cabinet in adjacent lab
- 0.5N NaOH - in hood
- Heptan - yellow cabinet in adjacent lab
- 37% HCl - in hood
- Pyridin (Merck 1.07463) - yellow cabinet in adjacent lab
- 2-chloroethyl chloroformate (CECF) (Fluka 23180) *store at 4°C* *toxic* - walk in fridge
- UltraPure Water
- Chloroform (Fisher C/4960/15) - in hood
- Heparin stabilized plasma or a standard

Methods

Before starting:

- Pull ≤10 samples to derivatize
- Turn on centrifuge and set temperature to 4°C
- Prepare beaker with soapy water and place in hood
- Pull rack (cabinet under gram scale) and glass screw-cap vials (in white bin and bag on top of shelf to the R of the hood)
- Label tubes w/ sharpie (do not write on white section of tube)
- All solutions are in clear aliquot bottles except for the CECF, see step #18

Step 1:

1. Per sample to be derivitized label 3 glass vials and 1 GCMS autosampler vial
   - One for each of the three steps
2. Weigh **first** set of empty glass vials **with cap** (100 x 16 mm screw cap)
3. Add 450 µl plasma or standard (heparin stabilized plasma), cap.
4. Weigh tube, record.

5. Add 100 µl internal standard/volume marker (2.5 mM [1,2-$^{13}C_2$]Sodium acetate, 1 mM [1,2,3-$^{13}C_3$]Propionic acid, 1mM [1,2,3,4-$^{13}C_4$] Sodium butyrate), cap.
6. Weigh tube, record.
7. Wearing **nitrile** gloves and lab coat working in the hood….Add 750 µl **2-C**hloroethanol and 750 µl Acetonitril. NOTE 2-chloroethanol fumes are highly **toxic**. Vortex at low speed, cap
8. Centrifuge 2550 x g for 30 min at 4°C in the **HOOD**. Balance via tube placement, no need to weigh. Start button = diamond with | ; when done, red light will be solid
   - Label autosampler vials (using tape), insert the inserts
     - Label with "MS" + lab notebook page + sample #
9. Transfer 1600 µl (2x 800µl) of supernatant (top layer) to new tube…put remainder in **waste bottle #1**
   - Use white and red pipettor; remove the outer shaft, PULL button to adjust
   - Avoid white/bottom layer

Step 2:

10. Add 20 µl of 0.5 N NaOH (small bottle in hood).
11. Add 2000 µl Heptan vortex 30 sec (vortex 2 at once, alternating which one is in the vortex).
    - Use LARGE 5ml blue pipettor and huge tips (in tip cabinet behind hood near door)
12. Leave sample for 10+ min (i.e. 15min) for phase separation.
    - While waiting, dump waste #1; place tubes and caps in soapy water in hood for later cleaning
13. Transfer 1300 µl (650µl x 2) of water phase (bottom phase) to new glass vial.
    - Use red and white pipettor; once tip is in the bottom phase, allow some bubbles to escape the pipet tip to remove any contamination from the upper phase
    - Pull bottom phase through top phase by holding the pipette tip at the very bottom of the tube

Step 3:

14. Add 10 µl 37 % HCl (small bottle in hood). - DO NOT CAP TUBE
15. Add 100 µl Pyridin. - DO NOT CAP TUBE
16. Vortex at low speed to mix.
    - Pull CECF from walk-in fridge (blue label)

17. Add 50 µl CECF (2-chloroethyl chloroformate) slowly and carefully **WHILE** vortexing at low speed for 3 to 5 sec (hold tube upright; DO NOT CAP TUBE, IT WILL EXPLODE due to liberation of gas [CO2]). This is the most critical step of the procedure. NOTE: CECF is toxic.
    - Use the mustard and blue 5-50µl Finnpipette; Return CECF to fridge
18. Add 3000 µl DI water and 100 µl chloroform, cap loosely
19. Vortex at low speed for at least 30 sec, cap tightly
20. Centrifuge 2550 x g for 10 min at 4°C in the hood.
    - While waiting, dump waste #2; place tubes and caps in soapy water in hood for later cleaning
21. Transfer 80 µl of the organic phase (BOTTOM, white/opaque) to autosampler vials with inserts for GCMS analysis, cap.
    - GCMS takes ~45-60min/sample; run overnight. (Appendix D.5)

Appendix Y – Gas Chromatography and Mass Spectrometry

Concept

Gas chromatography (GC) involves the addition of a liquid sample onto a chromatograph column, which consists of a coiled, thin, glass tube coiled to a diemeter of 30cm. In the column, the sample is vaporized and then returned to the liquid state as the column temperature rises. The boiling temperature of the liquid sample determines the rate at which the samples move through the column. As the sample enters the mass spectrometer (MS), ions are introduced to the sample by way of electric ionization. Ionized samples are presented to the MS ion detector. The output from the MS is a mass to charge ratio (m/z). The gas chromatograph separates the molecules based on size/boiling point prior to MS, which is important as two molecules could have the same m/z yet have different masses and charges. The sensitivity of GC/MS is negatively correlated with GC column diameter, which is important as there is a background signal that originates from the GC. Therefore, narrower and higher peaks allow for better detection of sample molecule signal.

Method

Derivatized fecal preparations and plasma were run on a Thermo Electron Polaris Q mass spec in tandem with a Thermo Electron Focus GC using XCalibur software (version 1.4). The column used was a Varian FactorFour capillary column VF-170ms (30m, 0.25mm, 0.25um). One µL of sample was loaded with inlet temp set to 225°C on a split ratio of 80 running a constant flow of Helium carrier gas set to 1.2mL/min. The GC was initiated at 75°C ramped at 5°/min to 135°C then at 40°/min to 225°C. The MS was programmed to run in positive SIM mode collecting in three consecutive segments m/z pairs for acetate (43,47), propionate (57,59), and butyrate (71,73) in that elution order. The processing method used to integrate the area under the curves for each m/z utilized the ICIS algorithm.

Appendix Z - Geneious® Alignment of *Fibrobacter* spp. and *Ruminococcus* spp. 16S rRNA sequences with published *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* primers

Fibrobacter spp. and *Ruminococcus* spp. sequences were aligned with the *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* primers previously described by Hastie *et al.(2008)* (Table 7).

Fibrobacter spp.

Contig_2284N45

A.GGCGGA.G.AT.GC.A.AG.CGGA.C.TGTA.CAA..T.ACCG.GGG.C.CCAA.CC.C.C.G.GA.CC.TG.C.AG.TCCGGAC
TGCAC.CTC.T....TG.GATAGTTCA.GGGGCAAGC.GGAATTCCTGGTGTAGCGGTGGAATG.C.GTAGAGATCAG.
GAAGA.ACACCGAT.GGCGAAGGCAGCTTGCT.GGGGAC.TAATC.GACGCTC.AT.GCT.C.GAAAG.T..GCG.G..G
T.....A.G.C.....A.A.....A.....C..A.G.G..A.....T.T.A.....G.....-.....---.....-...-..-..-..---.-...------..----------
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Geneious alignment w/ AJ496032 = ***93.8%*** *pairwise identity*

Ruminococcus spp.

Contig_2985N2

A.GGCGGG.A.TG.GT.A.AG.TCAG.A.TGTG.AAA..T.CTAT.GGG.C.TCAA.CC.C.A.T.AG.AC.TG.C.AT.TTGAAA
CTGCTG.TTC.T....TG.AGTGAAGTA.GGGGTAAGC.GGAATTCCTAGTGTAGCGGTGAAATG.C.GTAGATAT
TAG.GAGGA.ACATCGGT.GGCGAAGGCGGCTTACT.GGGCTT.TTACT.GACGCTG.AG.GCT.C.GAAAG.C..G
TG.G..G-.....-.-.-.....-.-.....-.....-..-.-.-..-.....-.-.-.....-.....-.....---.....-...-..-..-..---.-...------..----------
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Geneious alignment w/ AF030447 = ***92.2%*** *pairwise similarity*

Appendix AA - Comparison of RNA Extraction Methods

Concept

RNA quality can be measured by analysis of an electropherogram (fluorescence [y] x time [x]) produced by an Agilent Bioanalyzer. Smaller molecules pass through the Bioanalyzer faster than larger molecules. The following parameters are used to assess RNA quality

1. 23S and 16S Peaks

The 28S peak represents the large ribosomal subunit and the 16S peak represents the small ribosomal subunit (i.e. 16S rRNA). Therefor the 16S peak appears to the left (smaller; passes through the bioanalyzer faster) of the 28S peak. Fluorescence peak height represents quanity. There should not be peaks between the 16S and 28S peaks or "noise" as this represents RNA degradation.

2. 23S/16S Ratio

The 23S rRNA gene is a larger and more stable 16S rRNA. Therefore, the smaller the 23S/16S rRNA ratio, the less 16S rRNA gene degradation (Kerkhof and Kemp, 1999). A 23S/16S ratio of 1.7 indicates high quality RNA (Booijink et al., 2010).

3. RNA Integrity Number (RIN)

Fluorescent peaks of the 23S and 16S rRNA genes are evaluated and compared to fluorescence of smaller fragments from degraded RNA. The optimal RIN number is 10 represents intact RNA; an RIN of 1 represents RNA that has been completely degraded (Schroeder et al., 2006).

Objective

To determine the appropriate fecal preparation method and commercial kit for equine fecal microbial RNA extraction.

Methods

Table 34 below represents samples, RNA extraction kits, and protocol alterations used.

Results

RNA quantity and quality are presented in Table 34; electropherograms are presented in Figure 18. The Qiagen RNeasy® Mini Kit using the Fungal/Plant protocol with on-column DNase protocol as described in Appendix D.8 was chosen due to the clear 16S and 23S peaks on electropherogram (Figure 18) and RIN number.

Table 34 - *Comparison of Equine Fecal RNA Extraction Methods*

VBI ID	Horse ID	Sample Date	Sample Storage	Extraction Date	Bead beating	Extraction Kit	DNase	56°C	ng/uL	260/280	260/230	23s:16s ratio	RIN
1	16	6/4/11	LN2	9/12/2011	+	QIA	-		328.1	1.74	1.46	2.3	6.2
2	16	6/4/11	RNAlater®	5/17/2011	+	QIA	+		40.65	1.95	0.08	0	2.5
3	16	6/4/11	LN2	5/12/2011	+	ZR	-		1350.9	2.11	2.3	1.5	7
4	16	6/4/11	LN2	9/12/2011	+	QIA	+		52.14	1.73	1.32	0	1
5	16	6/4/11	RNAlater®	9/9/2011	+	QIA	+		121.05	1.75	1.44	0	2.1
6	16	6/4/11	LN2	9/12/2011	+	QIA	-		327.76	1.81	1.44	0.4	4.4
7	16	6/4/11	RNAlater®	9/9/2011	+	QIA	-		457.83	1.79	1.44	5.5	7.3
8	16	6/4/11	LN2	9/23/2011	+	ZR	+		323.28	1.90	1.16	0.5	4.1
9	16	6/4/11	LN2	9/23/2011	+	ZR	-		539.98	1.94	1.57	0	1
10	16	6/4/11	LN2	10/5/2011	+	QIA	+		348.97	1.73	1.16	0.3	5.1
11	16	6/4/11	LN2	10/5/2011	-	QIA	+		86.67	1.39	0.87	1	6.2
12	16	6/4/11	RNAlater®	10/19/11	-	QIA	+		109.63	1.48	0.96	0.8	6.5
13	5	6/9/11	RNAlater®	11/16/11	+	QIA	+	-	113.96	1.38	0.66	0.7	7.2
14	16	6/9/11	RNAlater®	11/16/11	+	QIA	+	-	20.6	1.21	0.50	0.0	2
15	5	6/9/11	RNAlater®	11/16/11	+	QIA	+	+	103.48	1.56	0.82	0.0	3.7
16	16	6/9/11	RNAlater®	11/16/11	+	QIA	+	+	41.29	1.32	0.65	1.4	8.4
17	5	6/9/11	RNAlater®	11/16/11	+	QIA	+	-	9.03	1.33	0.28	0.0	NA
18	16	6/9/11	RNAlater®	11/16/11	+	QIA	+	-	19.56	1.35	0.36	0.0	NA
19[a, c]	23	6/9/11	RNAlater®	12/5/11	+	**QIA**	+	+	98.78	1.59	1.05	1.5	8.4
20[b]	23	6/9/11	RNAlater®	12/5/11	+	**QIA**	+	+	43.53	1.41	0.71	0.5	7.6

QIA: Qiagen RNeasy® Kit; ZR: Zymo Research Soil Microbe DNA MiniPrep™ Kit

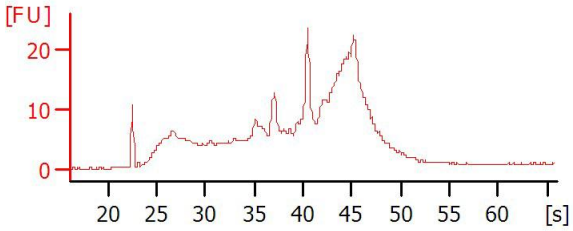
[a] *Samples washed 2x w/ PBS prior to DNA extraction;* [b] *the liquid portion after cell lysis (Appendix D.8) used for RNA extraction;*

[c] *Both the liquid and solid portion after cell lysis (Appendix D.8) used for RNA extraction*

Bolded samples were extracted using the RNA extraction protocol described in Appendix D.8).

Figure 18 - ***Electropherograms for samples 1-20***

1



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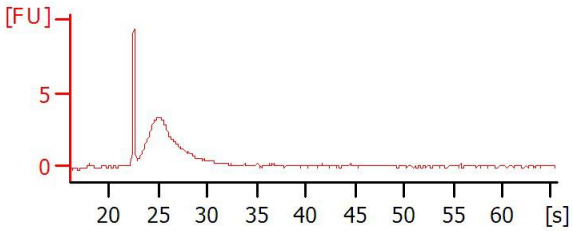

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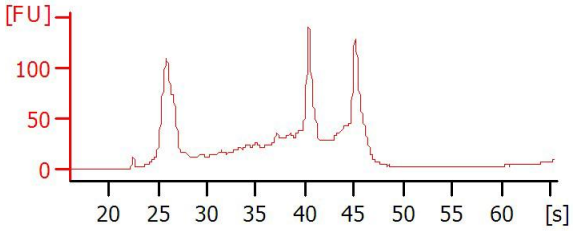

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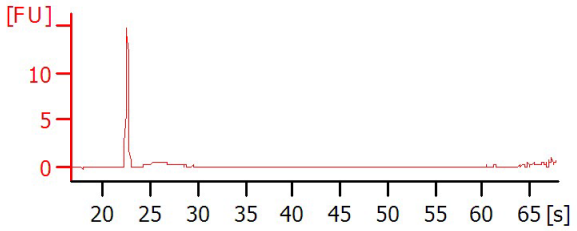

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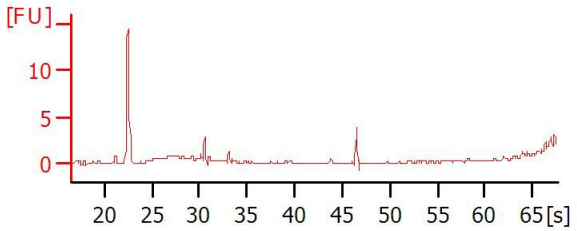

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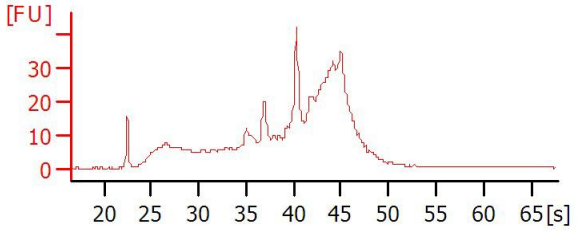

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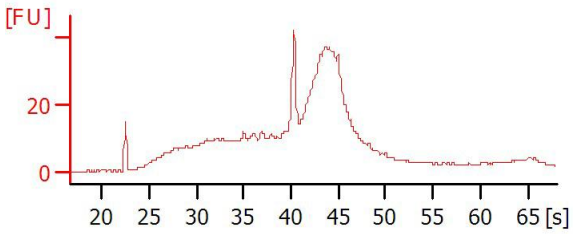
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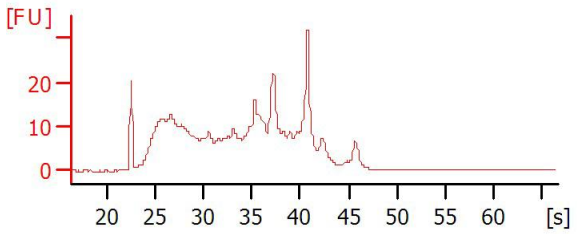
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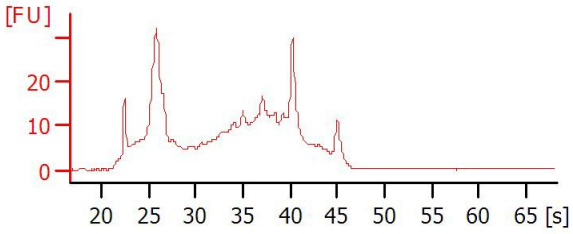
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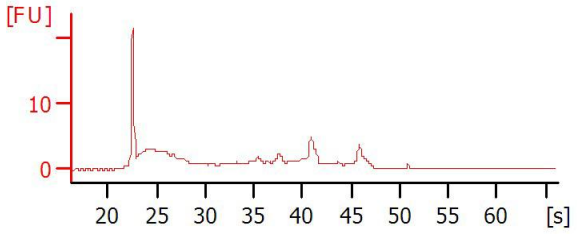
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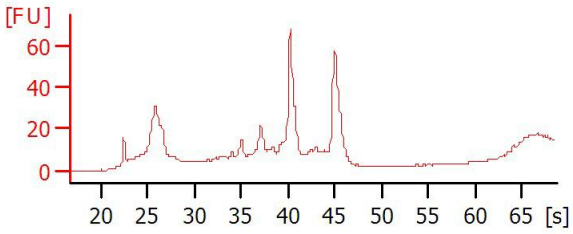
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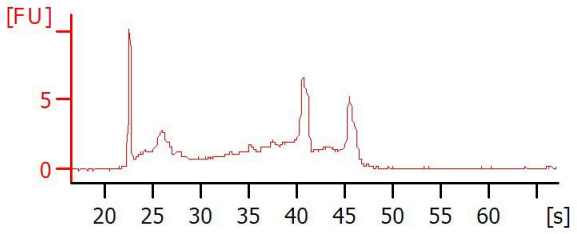
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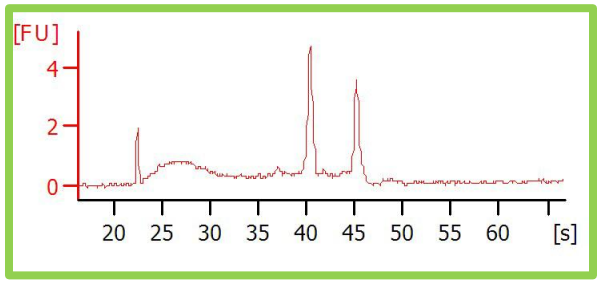

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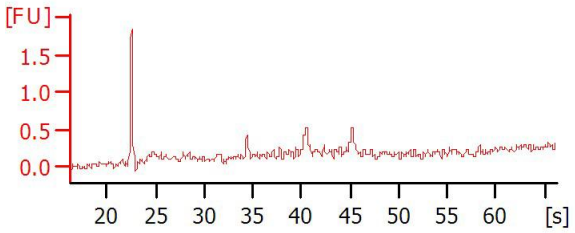

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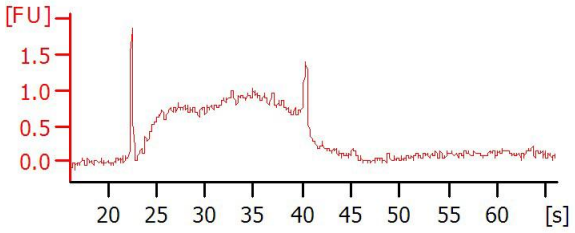

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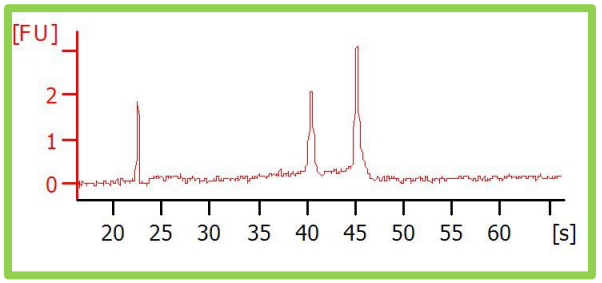

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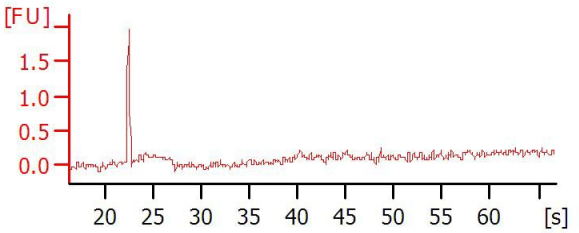

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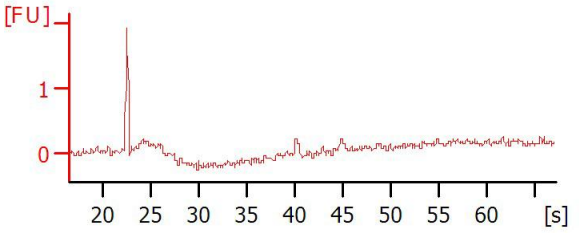

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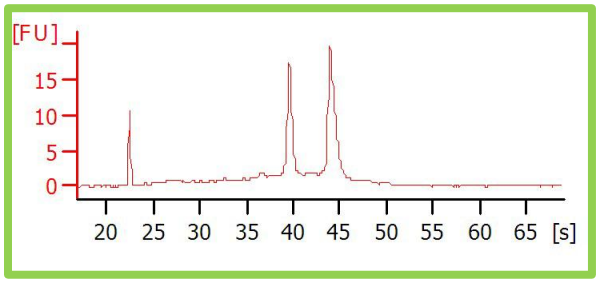
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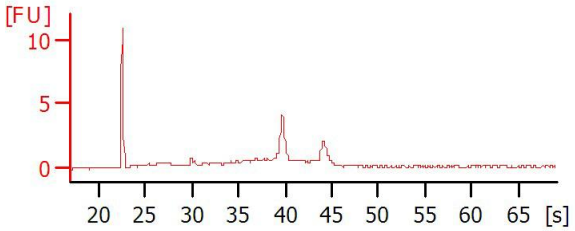
20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Appendix BB - Qiagen RNeasy® Mini Kit Fungal/Plant Protocol with On-Column DNase for Equine Fecal Microbial RNA Extraction

Materials

Item	Catalog #	Per sample
Eppendorph centrifuge (room temperature)	--	--
Vortex	--	--
Bead-beating vortex adaptor	Ambion AM 10024	--
70% Ethanol (bench top cleaning & protocol)	_	--
RNase AWAY®	_	--
Gloves		--
Tube rack	--	--
Cooler w/ ice	--	--
Timer	--	--
Sterile RNase-free 1.5ml tube	_	3
Sterile RNase-free 0.5ml tube (aliquot tubes)	_	3
Sterile scalpel blade	_	1
Pipette (10µL)	--	--
Pipette (200µL)	--	--
Pipette (1000µL)	--	--
Pipet tips (10µL)	Fisher 02707439	Sterile RNase-free
Pipet tips (200µL)	Fisher 02707430	Sterile RNase-free
Pipet tips (1000µL)	Fisher 02707404	Sterile RNase-free
RNeasy® Mini Kit	QIAGEN 74104	--
RNase-Free DNase Set (2-8°C)	QIAGEN 79254	--
QIA Shredder Mini Spin Column (lilac)	QIAGEN 79654	1
ZR IV-HRC Column (green top)	Zymo S6002-50	1
PBS (pH 7.4), 1X (40ml ddH2O + 4ml 10X PBS)	Fisher BP399	750µL
Beta-mercaptoethanol ß-ME (14.3M) (4°C)	Fisher _	Use to prep RLT buffer
Ethanol (100%/200proof)	Fisher _	500µL
Samples	--	--

BEFORE STARTING:

1. **Lay out tubes for RNA extraction (per sample; in order of use) and Label with horse # and date; tubes should be sterile and RNase free (SET OUT A PAIR FOR EACH SAMPLE, SEE CELL LYSIS).**
    - 1.5ml tube
    - QIA Shredder Mini Spin Column (lavender) + 2ml collection tube (label BOTH)
    - 1.5ml sterile tubes
    - RNeasy® spin column (pink) + 2ml collection tube
    - Collection tube
    - 1.5ml tube; label with horse #, collection date and "RNA/LN2"
    - ZR IV-HRC column (green) in a collection tube
        - Prepare by snapping off the base and centrifuging for 3 minutes at 8,000 x g, discard supernatant.
    - 0.5ml tubes (3/sample); label with horse #, collection date and "RNA/LN2"
2. **Attach vortex adaptor to vortex; set timer for 3 minutes**
3. **Turn on water bath to 56°C**
4. **Prepare buffer RLT!!!**
    - **a.** To a sterile 50ml tube add
        - **i.** 1000 µL buffer RLT x __ samples = _ µL
        - **ii.** 10 µL ß-ME x __ samples = _ µL
        - **iii.** Total 1000 µL /sample (2 tubes/sample; need 500µL each).
    - **b.** stable for 1 month at RT
    - **c.** RLT contains guanidine thiocanate to inactivate RNases
    - **d.** ß-ME denatures RNases via reducing disulfide bonds
5. **Prepare Buffer RPE (supplied as a concentrate) by adding ethanol.**
6. **Prepare QIA DNase stock; stable for 6 weeks at 2-8°C or 9 months at -20°C.**

Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550µL of the RNase free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not

vortex. Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial. For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at 20°C for up to 9 months. Thawed aliquots can be stored at 2-8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

7. **Prepare DNase**
    - **a.** To a sterile 1.5ml tube add
        - **i.** 140 µL buffer RDD x __ samples = _ µL
        - **ii.** 20 µL DNase (stock) x __ samples = _ µL
        - **iii.** Total 160ml/sample (2 tubes/sample; need 80µL each)
    - **b.** Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set. Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.
8. **Pull fecal samples from -80C and place in ice**
    - **a.** Allow samples to thaw just enough to allow for sample extraction; work in small batches (≤6 samples; even numbers are easiest).

Cell lysis

1. **To a 1.5ml tube, add 0.60-0.70g sample and 750 µL PBS.** Use a SCALPEL blade to remove frozen sample from the 50ml storage tube. **Centrifuge @ 6,000 x g for 1 minute, remove supernatant.**

   If the 50ml fecal "RNA" tube is cracked, parafilm the bottom to prevent RNAlater® leakage and transfer fecal sample + RNAlater® to a new 50ml tube after obtaining your 0.6-0.7g sample.
2. **Add an additional 750 µL PBS and vortex 3 minutes, high speed. Centrifuge at 10,000g for 1 minute.**
3. **SPLIT SAMPLE SUCH THAT: transfer supernatant to a 1.5ml labeled (a = liquid sample) tube but also use the solid pellet in step 2+ (b = solid sample).**

FOLLOW THE RNEASY MINI KIT PLANT and FUNGAL PROTOCOL:

4. **Add 450 µL to a tubes and 550 µL to b tubes of Buffer RLT WITH ß-ME to each 1.5ml tube (a & b) from step #1. Vortex vigorously for 1 minute.**
5. **Incubate at 56°C for 3 minutes.** Centrifuge "b" at 10,000 x g for 0.5min to pellet the solids (will clog the QIAshredder column), transfer the supernatant to the QIAshredder column.
6. **Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 10,000 x g. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.**

   It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very **small amount of cell debris will pass through** and form a pellet in the collection tube. **Be careful not to disturb this pellet when transferring the lysate** to the new microcentrifuge tube. The QIAshredder homogenizes, reduces viscosity of lysate, and shears high MW products (DNA etc) to improve RNA column binding.
7. **Add 0.5 volume (~500µL) of ethanol (100% / 200 proof) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.** Ethanol improves RNA binding to column (RNeasy® column).

   Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.
8. **Transfer the sample (usually 650 µL), including any precipitate that may have formed, to an RNeasy® spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at 8000 x g. Discard the flow-through.* Repeat to filter all supernatant from step #5.**
9. Add **350 µL Buffer RW1** to the **RNeasy® spin column**. Close the lid gently, and centrifuge for **15s** at **8000 x g** (10,000 rpm) to wash the spin column membrane. Discard

the flow-through.* Reuse the collection tube. RW1 contains ethanol to improve RNA column binding. The RNeasy® spin column has a silica membrane.

10. Add the **DNase I incubation mix (80 µL)** directly to the **RNeasy® spin column** membrane, and place on the **bench top** (20–30°C) for **15 min**. Note: Be sure to add the DNase I incubation mix directly to the RNeasy® spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.
11. Add **350 µL Buffer RW1** to the **RNeasy® spin column**. Close the lid gently, and centrifuge for **15 s** at **8000 x g** (10,000 rpm). Discard the flow-through.*
12. **Add 500 µL Buffer RPE to the RNeasy® spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g to wash the spin column membrane. Discard the flow-through.**

    Reuse the collection tube in step 9.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").
13. **Add 500 µL Buffer RPE to the RNeasy® spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g to wash the spin column membrane.**

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the RNeasy® spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
14. **Place the RNeasy® spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at 10,000 x g for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy® spin column after step 9.
15. **Place the RNeasy® spin column in a new 1.5 ml collection tube (labeled w/ horse ID, collection date and extraction date). Add 50 µL (30–50µl) RNase-free water directly to the spin column membrane. Wait 5 minutes then centrifuge for 1 min at 8000 x g**

to elute the RNA. RNA molecules are >200nt, thus enriches for mRNA (RNA molecules <200nt = tRNA, 5S rRNA, 5.8S rRNA [15-20% RNA]).

16. **Transfer eluted RNA into an ZR IV-HRC column and centrifuge for <u>1 minute</u> at <u>8000</u> x g. Combine a & b tubes at this point such that the filtered eluted RNA represents both the liquid and solid portion of each sample.**
    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.
17. **Check RNA concentration with NanoDrop**
18. **Standardize RNA concentration to 65-75ng/µL**
19. **Submit to VBI Bioanalyzer in 0.2ml PCR tube ~10 µL volume in a <u>0.5ml</u> tube (NOT a PCR 0.2ml tube per VBI website)**

Appendix CC - Evaluation of Cellulolytic and Acetogenic Gene Expression in the Feces of Mares fed Orchard Grass Hay

Introduction

Genomics only reveals some of the functional potential of the population: function by culture-based association or based on genes present in the genome. Transcriptomics is the study of gene expression. Much work has been devoted to understanding rumen microbial physiology and function. The horse relies heavily on the hindgut microbiome to metabolize fibers (i.e. cellulose) into products that can be used by the host (i.e. volatile fatty acids). Acetate is the dominant VFA produced by hindgut microbes and is a precursor for equine *de novo* fat synthesis (Suagee et al., 2010). Therefore, we set out to evaluate microbial cellulolytic and acetogenic gene expression in adult overweight mares vs. mares in moderate condition. Reverse transcriptase quantitative PCR was carried out as described in Section 6 using primers as previously described (Bera-Maillet et al., 2009).

To evaluate gene expression by way of reverse transcriptase quantitative PCR, a housekeeper gene must be used as an internal standard for each sample. Ideal housekeeper genes are those essential for replication and cell survival. In prokaryotes, the primary housekeeper genes are *gyrB*, *tuf*, *dnaK*, *rpoB*, and *recA*. DNA gyrase (*gyrB*), a type II topoisomerase, is essential for bacterial transcription and replication (Juste et al., 2008). DNA gyrase supercoils relaxed circular DNA and contains two subunits, GyrA and GyrB. Subunit GyrB is an ATPase essential for enzymatic activity of the GyrA, which cleaves and rejoins DNA during supercoiling (Akanuma et al., 2011).

Materials and Methods

One hundred thirty nanograms (2 µL) of template RNA was amplified in a reaction containing 12.5µL HotStart-IT SYBR® Green One-Step RTqPCR Master Mix Kit (75770 USB, Cleveland, OH), 1.3µL each of 16S rDNA forward and reverse primers (10µM; genes and primers listed below or in Table 7), 0.2µL M-MLV RT (USB #75783), 0.2µL RNase Inhibitor (USB #75782), 0.5µL ROX Passive Reference Dye (USB # 75768), and 7.5 µL nuclease free water (Amresco E476) in a 25µL reaction. The PCR protocol consisted of one cycle of reverse

transcriptase at 50°C for 5 min, one cycle of denaturation at 95°C for 2 min, 40 cycles of 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 3 min. Each reaction was prepared and carried out in biological and technical duplicates using an ABI 7300 (Applied Biosystems, Life Foster City, CA).

The genes targeted in the present study, their function, and previously used primers are listed below:

1) ***gyrB*** – housekeeper (Yamamoto and Harayama, 1995)
    a) UP1 5'-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3'
    b) UP2r 5'-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3'
2) ***endB*** - β-D glucosidase (Bera-Maillet et al., 2009)
    a) endBWF CGC TAC GAG AAG TGT GAC GA
    b) endBWR CTT CGA GGA TGA CGG AGA AG
    c) endBIF TTA CCA ACG GAG CGG TGT
    d) endBIR AGC CGA GCA TCA AAG TCG
3) ***acsB*** – acetyl-CoA synthase (Gagen et al., 2010; Rey et al., 2010)
        i) ACS_f CTB TGY GGD GCI GTI WSM TGG
        ii) ACS_r ARR CAW CCR CAD GAD GTC ATI GG
4) ***Bbct*** – butyrylCoa:acetateCoA transferase, acetogenic utilization (Louis and Flint, 2007)
    a) BCoATscrF GCIGAICATTTCACITGGAAYWSITGGCAYATG
    b) BCoATsrcR CCTGCCTTTGCAATRTCIACRAANGC

Results

The functional genes amplified poorly as compared to the 16S rRNA gene (Table 35).

Table 35 - ***Results from reverse transcriptase PCR***

Sample Name	**Target gene**	**Ct**
16	***gyrB***	**29.8**
16	***endB*** (BIF & BIR)	**27.9**
16	***endB*** (BWF & BWR)	ND
16	***acsB***	32.1
16	***Bbct***	ND
16	**16S** (338F + 518R)	8.8
16	No Primer	ND
16 – No RT	***endB*** (BIF & BIR)	33.0
16 – No RT	***endB*** (BWF & BWR)	ND
16 – No RT	***acsB***	ND
16 – No RT	***Bbct***	32.3
16 – No RT	**16S** (338F + 518R)	24.3
16 – No RT	No Primer	ND
NEG	***gyrB***	ND
NEG	***endB*** BWF & BWR	34.0
NEG	***acsB***	ND
NEG	***Bbct***	ND
NEG	**16S** (338F + 518R)	24.3

Discussion

Potential reasons for the lack of amplification include limited quality and/or quantity of RNA samples, the absence of these genes in equine feces, or lack of primer complementation. The 16S rRNA was successfully isolated from mare fecal samples; however, amplicons were not generated using the functional gene primers. Two potential explanations for lack of amplicon generation are: 1) inability to isolate mRNA from mare fecal samples and 2) lack of homology between mRNA present and functional gene primers.

Appendix DD - Section 6 Data Analysis with Post Hoc Exclusion of Horse 49 and 30

Table 36 - ***Mare body weight, BCS, and rump fat measurements taken on day 0 and 15 of the study and average dry matter intake throughout the study (d1-14).***

	Over-weight Mares (n=4)		**Moderate Condition Mares** (n=4)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
BW d0 (kg)	545		551		0.83
BW d15 (kg)	535		534		0.97
BCS (1-9/9)	7.2	7.0 – 7.5	5.3	5.0 - 6.0	<0.01
BMI = BW_{kg}/H_m^2	224	202-256	218	204-230	0.58
BMI = $BW_{kg}/(H_m*L_m)$	199	183-214	194	180-205	0.54
Rump Fat (cm)	2.17	1.94 – 2.49	1.52	1.05 – 1.91	0.04
Dry matter intake (% BW)	1.80	1.77 – 1.83	1.78	1.75 – 1.81	0.36

BW = Body weight (scale); BCS = body condition score; BMI = body mass index; H = height; L = length

Table 37 - ***Hay digestibility in overweight and moderate condition mares during d11-14 of the study.***

	Over-weight Mares (n=4)		**Moderate Condition Mares** (n=4)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
DE (Mcal/kg DM)	2.20	2.12 – 2.32	2.18	2.05 – 2.25	0.82
Digestibility (%)					
DM	55.3	52.6 – 57.7	54.1	51.6 – 56.1	0.49
NDF	59.1	56.7 – 61.5	59.0	56.4 – 61.7	0.94
ADF	53.7	51.5 – 56.2	53.5	50.1 – 57.3	0.94

DM = dry matter; NDF = neutral detergent fiber; ADF = acid detergent fiber

Table 38 - ***Volatile fatty acid concentrations in the feces (mg/g dry feces) and plasma (mmol/L) of overweight vs. moderate condition mares on d 11-14 of the study.***

	Over-weight Mares (n=4)		**Moderate Condition Mares** (n=4)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Fecal					
Acetate	8.83	7.43 – 10.75	8.02	6.43 – 9.57	0.45
Propionate	4.67	2.77 – 6.31	4.99	3.52 – 6.67	0.75
Butyrate	0.70	0.46 – 0.91	0.72	0.53 – 0.97	0.86
Plasma					
Acetate	1.57	1.43 – 1.64	1.37	1.22 – 1.47	0.04

Table 39 - ***Abundance (log_{10} copies DNA) of total bacteria, Firmicutes, and Bacteroidetes per gram of overweight vs. moderate condition mare feces.***

	Over-weight Mares (n=4)		**Moderate Condition Mares** (n=4)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	9.11	8.84 – 9.21	9.11	8.99 – 9.33	0.97
Firmicutes	8.90	8.62 - 9.09	9.03	8.87 – 9.26	0.33
Bacteroidetes	8.52	8.41 – 8.70	8.59	8.51 – 8.76	0.44

Bacterial abundance was determined by use of SYBR® Green primers.

Table 40 - ***Abundance (log_{10} copies DNA) of total bacteria, Ruminococcus flavefaciens, and Fibrobacter succinogenes per gram of overweight vs. moderate condition mare feces.***

	Over-weight Mares (n=4)		**Moderate Condition Mares** (n=4)		**Group**
	Mean	**Range**	**Mean**	**Range**	**p=**
Total bacteria	8.39	8.34 – 8.46	8.38	8.34 – 8.46	0.81
Ruminococcus flavefaciens	6.80	6.49 – 6.98	6.84	6.71 – 6.95	0.74
Fibrobacter succinogenes	5.24	5.07 – 5.46	5.56	5.42 – 5.77	0.032

Bacterial abundance was determined by use of TaqMan® primers/probes.

Discussion

DMI did not differ between groups in the present study; however, this study was not designed to evaluate voluntary DMI. Daily DMI is generally negatively correlated with digestibility, which is why we chose to remove the two mares with the lowest DMI from the study. One of the two horses with the lowest DMI had the highest digestibility for DM, NDF, and ADF. Similarly, Edouard *et al.*(2008) found no relationship between DMD and DMI in adult Standardbred geldings in ideal body condition although DMI was negatively correlated with NDF. The negative correlation between DMI and NDF is not a surprising finding as NDF represents hay bulk and positively influences satiety.

A difference in 0.02Mcal/kg hay DM in the 500kg horse fed 2% BW per day in hay (10kg hay DM per day) translates to an extra 73Mcal DE per year in the overweight horse. At about 23kg per BCS and 7Mcal per kg of adipose, each BCS in a 500kg horse contains about 161Mcal. Therefore, this difference in digestible energy could account for an increase in more than 1 BCS within three years.

Appendix EE - SAS Code for GIMMIX Procedure for Evaluating Body Weight (BW), Body Condition Score (BCS), Rump Fat, Digestibility, Volatile Fatty Acid (VFA) Concentrations, and Bacterial Abundance.

```
dm'clear log'; dm'clearoutput';
PROC IMPORT OUT= WORK.allaltVFA
DATAFILE= "P:\Private\BMVS PhD\CVM 2011\Data\CVM 2011 SAS ALL DATA.xls"
DBMS=XLS REPLACE; SHEET="SAS"; GETNAMES=YES; MIXED=NO;
    /*SCANTEXT=YES; USEDATE=YES; SCANTIME=YES;*/

options helpbrowser=sas;
ods graphics on;
ods rtf;

/* Horse_ID Age BW_d0 BW_d15 Ave_BWT_kg BCS Group rump_fat BMI_1 BMI_2 */

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model BW_d0 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model BW_d15 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model BCS = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
```

```
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model rump_fat = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

/* DMI_kg_BW DE DMD ADFD NDFD */

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model DMI_kg_BW = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model DE = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model DMD = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
```

```
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model ADFD = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model NDFD = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;


/* plasma_acetate fecal_acetate fecal_propionate fecal_butyrate */

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model plasma_acetate = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model fecal_acetatemg = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
```

```
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model fecal_propionatemg = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model fecal_butyratemg = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

/*          EUB_log10_g_manure_Shepherd8
            FIR_log10_g_manure_Shepherd9
            FIR_EUB_Shepherd9
            BAC_log10_g_manure_Shepherd12
            BAC_EUB_Shepherd12 */

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model EUB_log10_g_manure_Shepherd8 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model FIR_log10_g_manure_Shepherd9 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
```

```
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model FIR_EUB_Shepherd9 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model BAC_log10_g_manure_Shepherd12 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model BAC_EUB_Shepherd12 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model EUB_19_20 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;
```

```
proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model Rflav_19_20 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model Fsucc_19_20 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

/*          EUB_log10_g_manure_Shepherd21_22
            Rflav_21_22
            Fsucc_21_22 */

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model EUB_log10_g_manure_Shepherd21_22 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=allaltVFA plots=studentpanel;
  class group horse_ID;
  model Rflav_21_22 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;
```

```
proc glimmix data=allaltVFA plots=studentpanel;
 class group horse_ID;
 model Fsucc_21_22 = group;
 random horse_ID(group);
 lsmeans group;
 output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
 if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;


ods rtf close;
ods graphics off;


dm'clear log'; dm'clearoutput';
PROC IMPORT OUT= WORK.cDNA_DNA
DATAFILE= "P:\Private\BMVS PhD\CVM 2011\Data\CVM 2011 SAS ALL DATA.xls"
DBMS=XLS REPLACE; SHEET="SAS"; GETNAMES=YES; MIXED=NO;
     /*SCANTEXT=YES; USEDATE=YES; SCANTIME=YES;*/

options helpbrowser=sas;
ods graphics on;
ods rtf;

data cDNA_DNA_E;
      set cDNA_DNA;
      if horse_ID = 5 then FS1920_2122 = .;
      /*if horse_ID = 71 then FS1920_2122 = .;*/
run;

proc glimmix data=cDNA_DNA_E plots=studentpanel;
  class group horse_ID;
  model EUB1920_2122 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=cDNA_DNA_E plots=studentpanel;
  class group horse_ID;
  model RF1920_2122 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
```

```
proc sort; by student;
proc print data=potentialOutliers;
run;

proc glimmix data=cDNA_DNA_E plots=studentpanel;
  class group horse_ID;
  model FS1920_2122 = group;
  random horse_ID(group);
  lsmeans group;
  output  out=mydiagnostics  resid=residual  pred=predicted student=student;

data potentialOutliers; set mydiagnostics;
  if abs(student) > 3 ;
proc sort; by student;
proc print data=potentialOutliers;
run;

ods rtf close;
ods graphics off;
```

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