ABSTRACT

BOWMAN, MORGHAN ALEXIS. Effect of Diet and Restricting Time Allowed for Grazing on the Fecal Microbiome and Microbial Fermentation Parameters in Horses (Under the direction of Dr. Paul Siciliano).

Two experiments were conducted in October of 2016 to determine the effect of diet and restricting time allowed for grazing on microbial fermentation parameters and the fecal microbiome in horses. The first experiment (EXPT1) evaluated the effects of restrictive grazing for 8 hours overnight using grazing muzzles. Experiment 2 (EXPT2) evaluated the effect of an abrupt change in diet from hay to pasture and vice versa.

Eight mature stock-type geldings (587 ± 51 kg BW; Age 11 ± 1.69 yr.; ± SD) with maintenance only requirements were used in EXPT1. Horses were randomly assigned to control (CON, n = 4) or restricted (RES, n = 4) for a 14 d period. Control horses were allowed to graze continuously (24h/d); restricted horses were allowed to graze for 8 hours per day from 0800 to 1600 and were then muzzled from 1600 to 0800 the following day. All horses were grazed together in a 0.45 ha grazing cell designed to facilitate a dry matter intake of at least 3% of body weight per horse per day; horses were moved into a new cell of the same size every 3 days. On day 14 CON horses were brought into partially closed pens and offered free-choice grass hay for an additional 14 days during EXPT2. Bodyweight and fecal samples were collected on day 0 (turnout) and then every 7 days. Fecal pH was immediately recorded; the remaining fecal sample was divided and stored in a -80º C freezer for VFA and microbial analysis. Microbiome data was processed using MacQIIME (version 1.9.1). Alpha diversity was assessed using a parametric two sample t-test; beta diversity was assessed using ANOSIM (R 3.2.1). Differences in response variables (body weight, fecal

VFAs, fecal pH) and microbial relative abundances were evaluated using proc mixed (SAS 9.3) ANOVA for repeated measures design. A P-value < 0.05 was considered significant; P < 0.1 was considered a trend.

Body weight of RES decreased ($P < 0.05$) 19 ± 7 kg from d 0 to d 14 compared to an increase ($P < 0.05$) in CON of 22 ± 7 kg (treatment x time, $P < 0.01$). Mean fecal pH was 7.31 and 7.24 (± 0.019) for the CON and RES, respectively. Fecal pH not significantly affected by restriction or abrupt diet change; however the CON group tended ($P = 0.08$) to be higher. Fecal total VFA concentration decreased following the change in diet from hay to pasture (EXPT1), but did not differ following the change back to hay (EXPT2). Molar proportions were not affected by restriction, but differed significantly over time following a change in diet. A total of 3,720 OTUs were observed. Two of the following predominant phyla represented 80.7% of OTUs observed in both experiments: Bacteroidetes (47.45%) and Firmicutes (33.25%); Firmicutes tended to be higher in the control group and Bacteroidetes increased over time ($P < 0.05$). Principle coordinates analysis of unweighted and weighted UniFrac distances as well microbiota stability (Jaccard index) were different ($P < 0.05$) between treatments and over time during EXPT1 and EXPT2, respectively. Linear discriminant analysis effect size indicated an overrepresentation of *Fibrobacter* and *Clostridium* in RES horses while control horses had higher abundances of *Ruminococcus* and *Prevotella*. Restricting time allowed for grazing using grazing muzzles appears to impact fecal microbial profiles; however fecal fermentation parameters seem to remain unaffected over the short term. Additionally, abruptly changing the diet from hay to pasture (and vice versa) appears to impact both microbial profiles and fermentation parameters.

Effect of Diet and Restricting Time Allowed for Grazing on the Fecal Microbiome and Microbial Fermentation Parameters in Horses

by
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DEDICATION

This thesis is dedicated to husband and family for their unwavering support during these two years. Without the love, encouragement, and financial support from my husband absolutely none of this would have been possible.

BIOGRAPHY

Morghan Alexis Bowman, nee Hastings, grew up in Hope Mills, North Carolina, near Fort Bragg military base. She was not raised on a farm, nor owned or rode horses, but she knew from an early age that she wanted to work with animals and possibly become a veterinarian. She graduated from South View High School in 2011 and moved to Raleigh, North Carolina that August to attend North Carolina State University. As an undergraduate, Morghan majored in Animal Science with a double minor in Nutrition and Zoology. Her junior year was a turning point in her life when she decided against going to veterinary school, and discovered she had an interest in animal nutrition. After meeting with Dr. Paul Siciliano and becoming involved in equine research projects, she found her true passion. In the spring of 2015, after graduating with a Bachelor of Science, she continued her studies at North Carolina State University's Graduate School to earn her Masters of Science in Animal Science with a minor in Nutrition under the guidance of Dr. Paul Siciliano.

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INTRODUCTION

As a non-ruminant herbivore, horses have evolved with a complex gastrointestinal (GI) tract which specializes in the digestion and metabolism of plant structural carbohydrates into short chain (volatile) fatty acids (VFAs) for energy [1]. VFA production is achieved through the process of anaerobic fermentation by microbes existing symbiotically within the caecum and large colon or "hindgut" of the horse. Bacteria, along with protozoa and fungi constitute the microbial population (microbiota); their collective genomes compose what is known as the microbiome [2,3].

Compartments of the GIT including the mouth, stomach, small intestine, and hindgut have been studied to elucidate their unique microbiomes [2,4-8]. The microbiome of the hindgut, more specifically the fecal microbiome, has been extensively evaluated with the next-generation sequencing (NGS) techniques [4,6,9-17]. Research has shown associations between changes in the microbiota with laminitis [18], colic [4,13,19,20] , colitis [6], obesity [5], equine metabolic syndrome [17], and equine grass sickness [21]. Furthermore, dietary studies evaluating the addition of concentrate or starch proportions in relation to fiber also show shifts in the hindgut microbiota following diet change [22,23]. These studies confirm the complex relationship between diet, hindgut microbiota, and disease. Unraveling this relationship will lead to the understanding of the impact current management practices, such as those implemented on horses grazing pasture, have on horse health.

Horses at pasture can meet and often exceed their daily recommended digestible energy requirements [24]. This is especially true for horses with lower requirements such as those at maintenance, light work, or in early gestation. Regardless of feeding class, overconsumption of energy can contribute to an increased susceptibility of adverse health effects such as obesity,

insulin resistance, and pasture-associated laminitis [25-27]. Pasture restriction via grazing muzzles is a common method implemented by horse owners in order to regulate caloric intake and has been shown to be effective in maintaining [28] or decreasing body weight [29]. In addition to changes in body weight, restrictive grazing methods have been associated with changes in fecal fermentation parameters including pH, total VFA concentration, and molar VFA proportions [24,30].

The effects of an abrupt dietary change from grass hay to pasture (and vice versa) as well as pasture restriction on the fecal microbiome have not been evaluated to date. Understanding the potential consequences of feeding management practices on the microbiome and fermentation parameters can lead to the development of better management practices to regulate intake and body weight without negatively impacting horse health.

CHAPTER 1: LITERATURE REVIEW

Pasture Intake and Horse Health

Horses grazing pasture can meet and often exceed the NRC recommended daily digestible energy (DE) requirements; this is especially true for horses with relatively low requirements such as those at maintenance and light work [1]. When caloric intake exceeds energy expenditure or maintenance requirements, it becomes a contributing factor to over conditioning and obesity [26]. Obesity has been associated with a higher risk of developing disorders such as insulin resistance, laminitis, or equine metabolic syndrome (EMS) [25,31,32].

Laminitis is a debilitating disease resulting from inflammation and weakening of the epidermal laminae and subsequent disassociation from the hoof wall at the lamellar dermal-epidermal junction [27]. Under the horses' weight, this attachment failure can cause dramatic rotation of the distal phalanx which results in chronic mild to severe pain which may require the need for humane euthanasia. According to a United States Department of Agriculture survey, conducted in 2000, 54% of laminitis cases in the United States are pasture-associated [33]. Oligofructose (fructan) overload from lush spring to fall grasses is a well described constituent of pasture-associated laminitis [25,34]. Non-structural carbohydrates (NSC) in plants includes simple sugars, fructans, and starches [35]. These compounds are rapidly fermentable and when consumed in excess of an estimated threshold of 3 gKg^{-1} BW, the foregut cannot accommodate total digestion and therefore some carbohydrates proceed to the cecum and large colon where they are rapidly fermented [36]. This fermentation results in an accumulation and rapid absorption of lactic acid and other pro-inflammatory substrates leading to weakening of the laminae [25]. Induction of laminitis for scientific models occurs with a bolus of approximately 17 gKg^{-1} BW of starch or 7.5-12 gKg^{-1} BW of oligofructose [25]. The exact mechanism of how

this occurs is currently unknown. With the cost of veterinary care and potential loss of the horse, controlling pasture intake to mitigate the risk of obesity and other disorders is a top priority for horse owners.

A potential solution to this problem is to attenuate dry matter intake by developing a grazing strategy whereby DE intake meets, but does not exceed requirements. The common method employed by horse owners to regulate intake is pasture restriction by use of a grazing muzzle, allotted pasture, time out at pasture, or a combination of these methods. Although the efficacy of these methods and their effects on horse health over time continues to be evaluated; research has shown that pasture restriction can be an effective tool to regulate intake and body weight; however, there are also potential disadvantages.

The primary purpose for horse owners to implement pasture restriction methodologies is to regulate pasture intake. The intake of mature ponies out at pasture was found to be an average of 3.8% live weight [37]. To determine the effectiveness of grazing muzzles on ponies, Longland and colleagues studied the 3 hour intake of muzzled versus un-muzzled ponies in a cross-over design. The muzzles were shown to be effective as dry matter intake (DMI) of the ponies when restricted was recorded to be 0.14% of their live weight with an 83% reduction in DMI [38]. The effects of restricting time allowed for grazing on the DMI of adult horses were studied by Glunk et al. Horses were placed into groups and allowed to graze for 3, 6, 9, or 24 hours for 7 days over four weeks; every week the groups were switched to where all horses experienced each of the grazing times [24]. The total daily DMI was unaffected by treatment which means the DMI was similar regardless of the amount of turnout time. This suggests that the restricted horses made the most of their allotted time and were able to achieve an intake level near to those continuously grazing. Time of day also plays a role in developing a grazing program as NSC content in plants

has been shown to be higher during the evening when compared to morning hours [30,39]. Forage preference may also be a factor as horses were found to have difficulty consuming longer in comparison to shorter swards when muzzled [40]. This may not be an issue in regards to NSC intake as plants with shorter sward heights have been associated with a lower NSC content [41]. Additionally, forage species does not appear to impact the effectiveness of grazing muzzles when several cool season grasses were tested [42]. The intake of horses out at pasture can be regulated as shown in the grazing muzzle study; however, the time allowed and time of day for grazing should be considered when designing a grazing program as a means to regulate or reduce body weight.

Managing pasture intake below or at maintenance DE requirements influences body weight. Mature idle horses were muzzled for 12 hours overnight and allowed to graze for an 8 hour period in the study by Dowler et al.; horses maintained body weight over a period of 6 weeks [28]. Ponies muzzled for 10 hours per day over a 3 week period had a significantly smaller change in body weight when compared to an subsequent 3 weeks of un-restricted grazing; some ponies lost weight during the restricted period [43]. A study by Gill and colleagues used ten stock-type geldings to determine the effects of restricting the time allowed and space for grazing on body weight, body condition score, and serum insulin [44]. Restricted horses were allowed to graze 8 hours per day in a grazing cell containing enough herbage mass to facilitate an intake of 80% DE requirements for an average horse at maintenance based on the recommended NRC values. When compared to continuously grazing horses, the restricted group lost a statistically significant amount of body weight and reduced body condition score over time [44]. Serum insulin was also shown to decrease over time in these horses, indicating a positive

health effect [44]. These results demonstrate that weight reduction can occur on pasture when restricted by time and space allowed for grazing.

Adversely, pasture restriction has also been shown to increase dry matter intake rate (DMIR) and decrease the level of activity. Horses restricted by time and space were shown to numerically increase their consumption of dry matter over time from 11 to 32 g DM $\cdot$kg BW^{-1} $\cdot d^{-1}$ [44]. This is consistent with findings by Dowler and Glunk who also reported varying DMIR. Dowler found that restricted horses consumed approximately 55% of DE requirements in just the first 4 hours of grazing, and the rate subsequently decreased in the following 4 hours [28]. Additionally, behavior has also been shown to be impacted during pasture restriction studies. In a study by Fowler and colleagues, the activities of 12 mature horses were recorded when freely grazing and when muzzled. Horses were reported to spend 81 versus 41% of their time standing when muzzled and freely grazing, respectively [45].

Restricting time allowed for grazing with the use of grazing muzzles can be an effective method for regulating intake and bodyweight which subsequently decreases the risk for developing obesity, insulin resistance, and/or laminitis; however, it has been shown to increase the rate of dry matter intake which can alter hindgut fermentation parameters [24,30]. Understanding how the hindgut may be affected by management practices may provide an insight into the horse owner's role in the development and potential prevention of debilitating diseases such as laminitis.

Role of the Hindgut Microbiome

It is estimated that a horse at maintenance obtains approximately 30% of its energy requirements from short chain or volatile fatty acids (VFA) absorption; up to 80% of requirements with a diet high in fiber [46,47]. As a hindgut fermenter, VFA production occurs in

the cecum and large colon as a result of microbial digestion of dietary fiber and starch [1]. The symbiotic relationship between microbiota and equid can be defined as mutualistic, whereby the microbes thrive on substrates consumed by the horse and reproduce in an idealistic environment that facilitates growth; the horse receives a source of energy from digestion of otherwise indigestible feed, and is provided several B vitamins and vitamin K essential to the diet [1]. In a similar manner to ruminants, horses have evolved to depend on this symbiotic relationship to maintain their impeccable efficiency of nutrient digestibility and propensity as a non-ruminant herbivore.

Inoculation of the gastrointestinal microbiome in foals is thought to occur through consumption of maternal feces [48]. Establishment of microbiota begins rapidly after birth and significantly shifts up to 30 days of life then stabilizes [49]. Each compartment of the digestive tract contains its own unique microbiome, although similarities in microbiota have been reported between adjacent compartments [6,11]. Although gut microbiota is composed of many types of organisms (bacteria, fungi, bacteriophages, and protozoa) bacteria is the most heavily studied in equine literature [7]. The three major bacterial phyla within the cecum and colon of healthy horses have been extensively described as Firmicutes, followed by Bacteroidetes and Proteobacteria [2,4,11,12,15,50]. The species classified within these phyla, among others, are described by their function in substrate digestion. Fiber digesting or cellulolytic bacteria includes *Ruminococcus albus, Ruminococcus flavefacians ,* and *Fibrobacter succinogenes*; *R. flavefacians* was found by Julliand and colleagues to be the predominant cellulolytic species within the cecum [51]. *Streptococcus bovis* is an amylolytic bacterium, meaning it digests starch. Lactic acid producing bacteria (LAB) includes *S. bovis* and *S. equinus* as well as some *Clostridium* species; the genus *Lactobacillus* was found to represent the majority of LAB by Al

Jassium et al [52]. Lactate utilizers include some species under the genus *Megasphaera* and *Veillonella* [53].

Early culture based studies approximated a total of $25.9x10^8$ and $6.1x10^8$ bacteria per gram of contents in the cecum and colon of euthanized horses, respectively [54]. However, more recent estimates range from $10^{6.8}$ to 10^{11} per mL of content in the cecum and colon [7,18,55,56]. In addition to nutrient digestion and production, commensal bacterial populations also prevent colonization of pathogenic organisms in healthy individuals [57]. This occurs by the production of several antibiotic agents (metabolic by-products, exotoxins, bacteriocins) and adjustment of pH [58]. These compounds inhibit the colonization of other bacterial species; bacteriocins can be produced in vitro and are being evaluated as potential alternatives to antibiotics [58]. The role bacteria play in the physiological functions of equids is not completely understood; however, the gut microbiota has been reported to influence immunity and epithelial integrity in other models.

Experiments with germ-free mice demonstrate the role of the microbiota in immunity and intestinal integrity. Intestinal permeability was induced in wild type and germ free mice, the production of immune cells (CD4 T-cells and interleukin (IL)-17A) significantly increased in wild type mice as compared to germ free during exposure to the same pathogenic organisms [59]. Dysregulation of the immune cells is a proponent of local and systemic inflammation [60]. This interaction between the immune system and gut microbiota is poorly understood in horses among other species. Davison and colleagues noted the involvement of the intestinal microbiota of zebrafish in the regulation of gene expression in the intestinal epithelia through the upregulation and downregulation of transcription factors such as the HNF4A (hepatocyte nuclear factor 4 alpha) which is associated with inflammatory bowel disease in humans [61]. This association may be linked to the regulation of tight junction proteins which maintain the space

between enterocytes and the permeability of the intestinal epithelium [62]. In the horse, impaired tight junctions of the epithelial cells lead to what is commonly known as “leaky gut” syndrome where the permeability of the intestinal tissue is compromised and is thus susceptible to the influx and efflux of pro-inflammatory antigens, cytokines, toxins, bacteria and other luminal contents [60]. Systemic inflammation from a leaky gut is theorized to be a component to the pathogenesis of laminitis [27,34].

Microbiome research focuses on shifts in the microbiota associated with changes in physiological state, diet, and the onset of or during disease. These shifts are measured by taxonomic diversity, stability, and community structure through next generation sequencing analysis [63]. High variability within these measurements naturally exists between and within horses [7,15,16]. Currently, a “core” microbiota or a standard for normal healthy individuals has not yet been established; however, researchers in this field continue to build upon the foundation laid by earlier culture based studies. A laboratory out of Delaware State University is conducting an Equine Microbiome Project. Similar to the Human Microbiome Project, the goal is to process large quantities of samples and by horse information, such as diet, compare these samples for similarities in bacterial communities in order to better define a normal or core microbiome [64]. Characterization of a core microbiome may greatly impact preventative and therapeutic measures in equine medicine. Understanding the composition of a healthy microbiome gives a baseline for veterinary diagnostics as well as a standard for horse owners to strive for by adjusting their management techniques to prevent diseases associated with population shifts.

The Gut Microbiota and Disease

The relationship between the gut microbiota and health is a convoluted one as it is unclear if changes in the microbiota influence health, reflect it, or a combination of the two.

Divulging the connections between the microbiome and the onset of disease is a driving force behind many human and animal microbiome projects. Such developments can lead to expedited diagnosis, prevention, and treatment of disease. In equids, recent culture independent studies have reported population shifts in healthy subjects in comparison to those with chronic or acute diseases or induced models. Shifts in the gut microbiota have been linked clinical disease states such as laminitis, colic, colitis, and other equine aliments.

According to a 1998 study by NAHMS (National Health Monitoring System) the incidence of lameness in the United States was estimated to be between 8.5 to 13.7 events per 100 horses per year [65]. A more recent (2015) survey of U.S. populations determined that lameness was the cause of 7.4% of mortalities in horses 1 to 20 years old, and 7.6% in those older than 20 years [66]. Laminitis itself accounts for 7.5 to 15.7 percent of lameness cases [33]. Regardless of population statistics laminitis remains a debilitating, costly, and often deadly disease. There are many theorized causes of laminitis although exact mechanisms remain unknown. The association of the disease with microbial changes is a consistent topic within equine microbiome studies, reflecting the seriousness and need to understand the mechanisms behind the disease.

One of the earliest associations between the microbiota and the onset of laminitis was conducted in 1978 using culturing methods. Lactic acid producing bacteria (LAB) were shown to shift significantly following carbohydrate overload. Most notably *Lactobacillus* proliferated at 8 and 24 hours following administration of starch and wood flour mix [67,68]. *Streptococcus* populations decreased by 8 hours, then returned to near baseline numbers at 24 hours [68]. These results have since been repeated with NGS techniques in both oligofructan and cornstarch induced laminitis [18,69,70]. Increases in LAB are associated with a higher concentration of

lactate in the cecum [18]. Cecal lactate appeared to increase immediately following carbohydrate administration via a nasogastric tube, and continued to do so over a 36 hour period. Furthermore, cecal VFA concentrations decreased in response to increasing lactate concentrations, displaying an inverse relationship [18]. These results reflect the decrease in total bacteria populations as reported in the aforementioned study. The decrease in VFA production may be explained by the loss of bacterial diversity. Horses with chronic laminitis have a decreased abundance of the phyla Firmicutes and an increase in Verrucomicrobia with the most abundant bacteria being Streptococcus spp. and Clostridium spp [71]. The changes in LAB continue to be the primary focus of laminitis research; however, the proliferation and translocation of potentially pathogenic bacteria are postulated to contribute to the development of the disease as well. Pathogenic organisms can include both Gram-negative and Gram-positive bacteria, the growth of which appears to occur in the cecum of these models with no evidence of bacterial translocation in extra-intestinal tissues [69]. The latter finding would mean that the bacteria may not move from the hindgut to other tissues and thus produce metabolites within the tissue which theoretically influences inflammatory response. In a similar study, Moreau and colleagues observed increases in *Veillonella* sp. and *Seratia* sp., which were not cultured in the earlier study [70]. These studies have shown significant taxonomic differences between normal (non-laminitic) horses and those undergoing induced laminitis which provides an insight into microbial changes during the development of the disease.

Additionally, changes in lactic acid producing bacteria populations are reflected by a significant decrease of cecal pH from near neutrality 6.5-7 before carbohydrate administration to around a pH of 4 [68,72]. Carbohydrate overload increases the rate of fermentation and lactic acid production, an accumulation of which causes the reduction of pH in the cecal fluid [34].

Rapid decrease in pH results in the decimation of several populations of bacteria that are unable to survive in such an environment. Following death, these bacterial cells release their contents into the lumen and subject the mucosa epithelia to endotoxin or exotoxin derived damage [34,73]. Such damage to the epithelia may cause leakage of the gut contents into the peritoneal cavity, causing endotoxemia and subsequent systemic inflammation [60]. This cascade of effects being just one of the several hypothesized etiologies of laminitis.

Second to lameness, the incidence of colic was estimated to be 4.2 events per 100 horses per year [65]. A 2005 survey of U.S. operations showed that 10.4% of horses at an age of 6 months or older became afflicted with colic [74]. With an approximated cost per event at $160 and a leading disease mortality rate of 31.2% in horses 1 to 20 years old, colic remains one of the top concerns for horse owners and equine practitioners alike [75]. Similarly to laminitis, colic is also associated with dietary change and starch overload [25,27,34]. As supported by the carbohydrate overload model studies, diet can impact the gut microbiota as discussed later in this review. Therefore, colic attributed to a change in diet can theoretically be caused by or result in changes in the hindgut microbiota and associated changes in fermentation parameters. Fecal analysis of pregnant mares that later developed postpartum colic showed that all 7 subjects had a relative abundance of Firmicutes less than or equal to 50% and an increase in Proteobacteria [76]. Additionally, horses admitted to the hospital for non-surgical colic had a greater number of sequence reads as compared to the same individuals post colic; diversity appeared to return following the colic episode, although diversity calculations were not reported [13]. Similar results were reported in horses with undifferentiated colitis which experienced an increase in the relative abundances of Bacteroidetes and Proteobacteria, while Firmicutes and members of the class *Clostridia* decreased as compared to healthy controls [6]. Although not directly related to

colic, horses hospitalized with diarrhea possessed similar microbial profiles to the aforementioned studies in addition to an increase in the phyla Verrucomicrobia and overall lower microbial diversity [77]. It appears that the microbial profile of horses with gastrointestinal diseases such as colic, colitis, and diarrhea are similar and such changes in bacterial phyla may be indicative of the onset of GI disorders. Additional studies are needed before a clinical analysis can be created.

Other equine ailments including grass sickness and obesity have also been explored in the microbiome field, although very few studies have been published on these diseases in relation to the gut microbiota in horses. Equine grass sickness (EGS) or equine dysautonomia is a neurological disorder primarily seen in grazing horses whereby the horse experiences nerve damage and subsequent enteric paralysis [78]. The acute form of EGS can halt intestinal peristalsis and cause death in a short time frame. The exact cause of EGS is unknown and continues to be researched although is postulated that bacterial derived toxins may be involved. A culture analysis of fecal and ileal samples of horses with chronic EGS reported higher clostridial counts and number of clostridial species found (1 versus 14 for healthy controls and EGS horses, respectively) compared to healthy controls [79]. NGS analysis of fecal samples from EGS and healthy horses showed similar results to horses with gastrointestinal disorders where Bacteroidetes increased and Firmicutes decreased [21]. No changes in Clostridia were reported in this study although the genera *Veillonella* increased in abundance. This discrepancy between the two studies could potentially be attributed to the method of bacterial analysis whereby the culture could have been contaminated or the sequencing may not have been fine or specific enough to determine relative abundances of those species previously identified. The similarity of these results to those of GI disease illustrates the impact of EGS on the GIT.

Obesity is a growing concern for developed countries as it was estimated in 1998 that 1.4% of horses in the U.S. were considered obese [80]. Although the current prevalence in the United States has yet to be determined, it can be assumed higher as a 2015 study in Great Britain reported 26% of horses to be obese [81]. Additionally, of the obese horses and ponies in the study, 21% presented with a history of laminitis and 16% experienced at least one episode [81]. Obesity is classified as a systemic inflammatory disease and as such increases the risk of developing other disorders like insulin resistance, laminitis, and equine metabolic syndrome [26]. The most compelling evidence of the involvement of the gut microbiome in the development of obesity is shown in germ free mouse models. The transfer of microbiota of mice with diet induced obesity in germ free recipients resulted in increased fat deposition in those subjects. Conversely, when transferring the microbiome of lean mice into obese mice, insulin sensitivity was shown to significantly improve [82]. However, mice given a high fat diet supplemented with the feces of their lean counterparts gained more weight compared to those given just the high fat diet; bacterial diversity as well as species richness increased only in mice given the supplement [83]. In dogs, lactic acid producing bacteria were numerically higher in obese individuals; however, when compared to lean counterparts, the relative abundances of bacteria were not significantly different [84]. Interestingly, non-obese dogs fed an ad libitum diet showed a significant increase in the order *Clostridiales*. This may suggest a role of microbiota in relation to diet and the onset of obesity. To date, there are no studies comparing the microbiota of obese horses to normal controls or the impact of diet or exercise induced weight loss. However, one study by Weese and colleagues evaluated the microbial profile of horses with equine metabolic syndrome to healthy individuals fed similar diets [17]. Equine metabolic syndrome (EMS) is characterized by obesity (increased adiposity), insulin resistance, and laminitis [85]. Horses

clinically diagnosed with EMS had lower microbial diversity and decreased representation of members from the *Fibrobacter* genera in the feces compared to healthy controls [17]. Members of the *Fibrobacter* genera are described as acid-intolerant and thus it was hypothesized that an increase in LAB, and subsequent decrease in pH, lowered the *Fibrobacter* counts in the EMS horses as seen in the carbohydrate overload models [86]. Obesity appears to be associated with a decrease in bacterial diversity and thus may affect the relative abundances; however, the interaction effect between diet and the microbiota, if it exists, has not been evaluated.

Although microbial changes in disease states have been documented, the mechanisms by which they occur and the overall effect on the gut microbiome are not completely understood. Microbiome studies have shown significant taxonomic differences between healthy horses and those induced or in a disease state which provides an insight into the development and effect of the disease on gut homeostasis. The association of the microbiome, diet, and risk factors for disease needs to be evaluated further in equine studies before the development of clinical microbial assays can occur.

Diet and the Gut Microbiome

Microbiota shifts have been reported to occur due to the sensitivity of factors such as the onset of disease, stress level, and diet [5,6,13,34,87]. Following disease, the effect of diet is the most thoroughly studied subject in this field as it is easily manipulated. The results of dietary studies provide a greater understanding of the contribution of bacterial communities in microbial digestion and the role of management in the health of equids. Furthermore, divulging the impact of diet composition and the effects of an abrupt change in diet on the microbiota can contribute to our understanding of the relationship between diet and associated diseases such as laminitis and colic.

Diet composition, whether high in fiber or starch, is an important determinant in feed selection. The most recent publication from the Delaware University Equine Microbiome Project described the evaluation of fecal samples from 184 horses in conjunction with dietary information with diets varying from pasture to hay to concentrate mixes. The profiles of pasture fed horses were found to be significantly different from those consuming a diet of hay/concentrate whereby the genera *Christensenellaceae*, *Oscillospira*, and *Prevotella* were reportedly higher in abundance; *Streptococcus* and *Lactobacillus* counts were different in horses consuming hay/concentrate diets, although these values were not fully reported [64]. Lactic acid producing bacteria counts appear to reflect a diet high in starch as shown in carbohydrate overload models [34].

The characterization of bacterial communities in feces of forage fed horses showed the largest relative abundance belonging to the phylum Firmicutes (43.7% of total sequences) followed by Verrucomicrobia (4.1%), Proteobacteria (3.8%), and Bacteriodetes (3.7%) [12]. Willing and colleagues found that horses maintained on a forage-only diet possessed a more stable fecal bacterial community structure over a sampling period of 29 days in addition to lower counts of LAB when compared to those fed concentrate-only diets [9]. Similarly, LAB, Bacteroidetes, and Lachnospiraceae were found to significantly increase in horses fed concentrate compared to grass-only diets [86]. These results continue to support the idea that diet high in starch can attribute to microbial dysbiosis; however, Murray and colleagues noted a minimal effect of feeding a high or low starch diet whereby diet did not impact cellulolytic or amylolytic bacterial counts, VFA, or lactate in the feces [88]. These conflicting results may be due to the type of diet fed as the high-starch mix was in a 50:50 ratio to mature grass hay which would theoretically contain a large amount of fiber and thus could have potentially thrown off

the diet effect. Furthermore, no effect was found on colonic bacterial concentrations, VFA, or pH of horses fed different processed forms of barley as a source of starch [89]. Additionally, Dougal et al investigated three high fiber diets, two of which were supplemented with starch and oil. Similarities of the fecal bacterial communities were observed between all three samples and were designated as a "core". The high fiber fed group had the largest core which was dominated by Firmicutes, followed by the oil supplemented group, and lastly the starch supplemented group had the smallest associated core [90]. The influence of high fat diets on gut microbiota need to be evaluated further.

It is common knowledge in both livestock and companion animal management to slowly transition when implementing a new diet. An abrupt change in diet can cause severe GI upset including colic and diarrhea. Horses fed a hay based diet with a sudden inclusion of varying amounts of rolled barley had an increase in Lactobacilli and Streptococci in both the cecum and colon following diet change [91]. Interestingly, a decrease of acetate and butyrate to propionate ratio was observed in addition to an increasing concentration of lactate; however, pH remained stable. The inverse relationship between VFA and lactate concentrations was seen in carbohydrate overload models, but with a more dynamic change in fecal pH (from neutral to around 4) [68,72]. This may indicate that a greater amount of starch or a different source may be needed to induce microbial changes associated with the induction of laminitis. An abrupt change in diet from hay to grass silage and haylage showed similar results whereby colon and fecal bacterial counts, VFA concentrations, and pH remained unaffected following 28 hours after the change [92]. However, Lactobacilli counts significantly increased in horses switched to the silage diet on day 21 and Streptococcus was observed to decrease in a greater amount in the haylage fed group. Although an immediate effect following dietary change was not observed, the

weekly observations of bacterial counts in the colon did differ 3 weeks following the abrupt change, suggesting a longer impact of diet change. The size and frequency of meals was also found to impact the cecal microbiota whereby one large meal had a greater influence on the bacterial communities when compared to three small meals given throughout the day [93].

As obesity and pasture-associated laminitis remain a growing problem, manipulating the hindgut microbiota through the diet appears to be a viable option for disease prevention or treatment. The effect of an abrupt change in diet from grass hay to pasture and vice versa on the fecal bacterial community has not been evaluated. Furthermore, the effects of restricting time allowed for grazing on the fecal microbiome have not been characterized. Understanding the effect of pasture restriction may also allude to the impact of weight loss on bacterial counts and vice versa. The significance of management in the complex relationship between diet and the microbiota needs to be explored in order to develop new or better current practices to enhance horse health.

Chapter II: Materials and Methods

The following experimental protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC Protocol 16-178).

Animals and Experimental Design

This study was conducted during the month of October 2016 at North Carolina State University Equine Research Unit located in Raleigh, NC. Eight mature stock-type geldings with maintenance only requirements, ranging in age of 7 to 18 y with an average body weight of 584 ± 55 kg (mean ± SD), were used in a four week (28 d) repeated measures experimental design to determine the effect of diet on the fecal microbial profile and other fecal parameters (fecal pH and VFA profiles). Prior to this experiment, all horses were used in an intake study where they

were offered free choice mixed grass hay for 36 d [94]. The intake experiment ended on day 0 of this study.

Experiment 1

Horses were randomly assigned to one of two treatment groups for a period of 14 d: 1) 24 h continuous grazing or control (CON), n=4; 2) 8 h grazing in the morning/late afternoon (RES), n=4. The RES group were restricted using standard basket grazing muzzles (Best Friend Equine Supply, New Holland PA) during the time of 1600 to 0800 EST. Grazing muzzles contained open holes which allowed for ingestion of feed and water. Horses had access to water and a trace mineral block (Morton 50 lb. Trace Mineral Block).

Experiment 2

Following collection on day 14, the control group were brought into the barn and randomly assigned to four partially covered stalls with an automatic water source for the second experiment (14 d); the RES group was released from the study. During this period, CON horses (n=4) were offered free choice mixed grass hay, and had access to water in addition to a trace mineral block. Hay rations were initially calculated as a percentage of 2.0% BW for each horse then adjusted according to refusal weight; each ration was weighed using a digital scale (Salter-Brecknell-PS150; precision = ± 0.1 kg). Refusals were collected and recorded to ensure horses were fed ad libitum.

Pasture Measurements

During the first experimental period, all horses were contained in the same area and exposed to the same pasture. The 3.16 hectare pasture was split into four 0.45 hectare grazing cells which were created using temporary fencing and connected to an electrical current (land area was estimated using Google Maps Area Calculator Tool). Horses were allowed to graze in

each cell for approximately 3 days, then moved into a new 0.45 hectare cell. Each cell was designed with enough herbage mass to facilitate a dry matter intake of at least 3% body weight per horse per day. Herbage mass was calculated using a falling plate meter [95]. The plate meter was constructed with a plexi-glass disc attached to a polyvinyl chloride sheath which was fitted loosely over a metal rod as described in Glunk et al [24]. The rod was labeled with a maximum height measurement of 20 inches (50.8 cm). Compressed sward height measurements were taken by lifting the sheath up to the maximum height and then releasing it on a randomly selected canopy. Forty compressed sward height measurements were obtained starting from one corner of the grazing cell and following a serpentine pattern to the opposing end of the cell. Measurements were taken approximately 10 steps apart with the meter being placed down on step 11; all measurements were taken without visually assessing the canopy to avoid bias. For calibration, nine additional measurements were randomly taken from the cell to represent three heights of high, median, and low canopies based off of the prior 40 measurements. A 0.25 x 0.25-m frame was placed around the selected canopy and the plants within this boundary were harvested and placed into pre-weighed labeled burlap sacks. The initial wet weight of the samples and sack was recorded. All sacks were placed into a 60ºC drying oven for approximately 72 hours to determine the percentage of DM. Samples were weighed every 24 hours; drying was considered completed when weights changed very little (±0.2 g) or no longer fluctuated. The dry weight was divided by the initial wet weight to obtain the percentage DM. These values were regressed against the compressed sward heights to produce an equation to predict the amount of DM available in the respective cell.

Additionally, the botanical composition of each cell was determined by the step-point method prior to each transition. This method begins at the corner of the grazing cell where an

observer takes 10 steps toward the opposing end of the cell and identifies the plant species which touches a predetermined point on the shoe. Fifty observations per cell were recorded in a serpentine-like pattern across the cell. Relative percentages were calculated by doubling the total number of each observed species. Pasture samples were also collected prior to each movement into a new cell. Samples were taken from plants of which the horses were observed grazing. Samples were sealed in bags and stored in a -20ºC freezer until chemical analysis at a later date. Chemical composition testing was conducted by the North Carolina Department of Agriculture Forage Testing Laboratory in Raleigh, NC. Forage testing included DM, CP, fat, NDF, and ADF.

Measurements and Sample Collection

Body weights were measured prior to collection on each sample day using an electronic scale (Smart Scale 200, Gallagher Animal Management, USA; precision = ± 1 kg). Fecal samples were collected every seven days including pretrial (day 0) for a total of 5 collections. Rectal grab-samples were obtained at 1400 each collection day with the exception of day 0 and 7 which were taken at 0900. In the event that a horse defecated prior to collection, samples were carefully collected from the top of the fecal pile to avoid environmental contaminants. Immediately after collection, fecal samples were thoroughly mixed within their respective labeled bags and subsamples were placed into three sterile flip top bags per horse. The first and second bags were filled with approximately 20 g of feces for DNA analysis. The third bag was filled with approximately 50 g of feces for VFA analysis. DNA and VFA Samples were placed into a 35°F (1.6°C) refrigerator until transport to campus at a later time the same day. These samples were transported on ice and stored in a -80°C freezer until analysis at a later date.

Fecal pH

Fecal pH was analyzed immediately following each collection. Fifty grams of the remaining sample for each horse was individually and thoroughly mixed with 50 g of deionized water then allowed to equilibrate for 5 minutes [96]. Measurements were taken in triplicate using a Symphony pH portable meter (VWR International, Batavia, IL) which was calibrated with a two-point calibration (pH 4.01 and 7) prior to each use according to the instrument instruction manual to establish accuracy of measurements.

VFA Analysis

VFA analysis started with sample preparation for GLC (gas liquid chromatography). Fecal samples were removed from the -80ºC freezer and allowed thaw to room temperature over a period of 3 hours. Approximately 20 grams of feces from each sample was weighed and placed into 50 mL conical vials. This process was carried out in duplicate whereby each sample had two conical vials with 20 grams of feces in each, respectively. Ten milliliters of deionized water was added to each vial; vials were vortexed for 10 seconds to mix and then stored in a -10ºC fridge overnight. The following day, samples were centrifuged at 1050 rcf (2500 rpm) in the Sorvall centrifuge for 15 minutes. One milliliter of supernatant was transferred from each sample into individual 1.5 mL microfuge tubes; 200 µl of MIS (25 % meta-phosphoric acid with internal standard, 2-ethylbutyric acid) was added to each tube with an internal standard for calibration. Samples were stored in a -80ºC freezer overnight; once thawed the following morning, samples were centrifuged at 21,000 rcf (15,000 rpm) for 10 minutes. Clean supernatant was transferred into individual GC vials to be analyzed by GLC. The gas chromatograph was equipped with a with Varian CP-8400 auto-sampler. During analysis, the auto sampler used a 10 µl gas tight syringe (Hamilton Co., Reno, Nevada) to add one microliter from each vial to the column. VFA

data was analyzed using a Varian software program. Final VFA concentrations were obtained by multiplying the output by the dilution factor to account for the initial dilution.

The DM fraction was determined by weighing approximately 6 grams of feces on aluminum plates; this process was also run in duplicate whereby two aluminum plates per sample held 3 grams of feces each. The initial weight of each plate and sample was recorded. The plates were then placed into a drying oven set to 100ºC for approximately 3 days. Following the drying period, all samples were placed into a desiccator for 20 minutes to cool before obtaining the final dry weight. DM fraction was calculated by dividing the finial by the initial weight.

DNA Extraction

DNA was extracted from 0.25g of each fecal sample using a QIAmp PowerFecal DNA kit (Qiagen Bioinformatics, CA) according to the manufacturer's procedure. Briefly, samples were removed from the -80ºC freezer and allowed to thaw for approximately 30 minutes and added to the provided dry bead tubes, then homogenized. Following column binding and wash steps, DNA was eluted with 75 µl of elution buffer. DNA quantity and quality were determined using a Nanodrop 1000 (Thermo Scientific, Raleigh, NC) and gel electrophoresis, respectively. DNA samples were stored in a -20ºC freezer until use.

Library Preparation

All library preparation steps were carried out according to the 16S Metagenomics Sequencing Library Preparation guide for the Illumina MiSeq system (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). For each sample the V3 and V4 hypervariable region of the 16S rDNA was amplified using an advantage PCR kit (Clontech Laboratories, CA) for the manufacturer's

instructions using the following primers:: forward primer: 5'-TCGTCGGCAGCGTCA GATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG -3' and reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC -3 according to Klindworth et al [97]. These primers were designed with the following overhang adapter sequences for compatibility with the Illumina indexing system: forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAA GAGACAG-[locus- specific sequence] and reverse overhang Reverse overhang: 5'GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAG-[locus- specific sequence]. All PCR reactions were carried out using a Bio-Rad T100 thermocycler (Bio-Rad, Hercules, CA); following Illumina's recommended conditions. Amplicons were purified using AMPure XP Beads (Illumina), washed in 80% Ethanol, and eluted with 10 mM Tris pH 8.5. DNA quantity and quality were determined using a Nanodrop 1000 (Thermo Scientific, Raleigh, NC) and gel electrophoresis, respectively. A second amplification step was carried out using the Nextera Library Preparation Kit (Illumina; San Diego, CA) with the included index primers following the manufacturer's instructions. Amplicons were then purified using AMPure XP Beads (Illumina) as described above. Library integrity and quantity was then determined were determined using a Bioanalyzer DNA 1000 chip at the NCSU Genomics Services Laboratory (GSL: North Carolina State University; Raleigh, NC). Libraries were then pooled at a final concentration of 10 nM and sequenced on a MiSeq platform (300bp, paired-end, v3 chemistry) (Illumina; San Diego, CA).

Sequence Analysis

Sequences were analyzed using MacQIIME (version 1.9.1.) [98]. The following commands were used based on the QIIME workflow according to Navas-Molina et al. [99].

Sequencing data received from the GSL were demultiplexed resulting in two fastq files per library as shown in figure 1 below.

Forward read S0_L001_R1_001.fastq.gz

Reverse read S0_L001_R2_001.fastq.gz

Fig. 1 Example of fastq files from Illumina sequencing

Paired-end reads were then joined using the multiple_join_paired_ends.py command, making a single file per library. The samples were then condensed further into a single seq.fna file using the multiple_split_libraries_fastq.py command. Sequences were clustered at a 97% similarity threshold by picking operational taxonomic units (OTUs) against a closed reference (Greengenes 13_8 database) with UCLUST (pick_closed_reference_otus.py) [100]. Closed reference OTU picking allows for quality filtering as sequences are compared to a known reference. Sequence reads that do not match this reference are excluded from the OTU table and subsequent downstream analyses. In this case chimera checking was not used due to the nature of closed reference picking. Core diversity analysis (core_diversity_analyses.py) of the OTU (BIOM) table simultaneously calculates alpha, beta, and taxonomic diversity using the mapping (.tsv) file containing sample metadata such as sample I.D, treatment group, and day (figure 15). This step includes rarefaction commands and was carried out at an even sampling depth based on the lowest sequence count per library (15,000 reads) within the OTU table in order to rarify (normalize) the libraries for diversity analyses. Microbiome analysis workflow used in this study

is shown in figure 2 below.

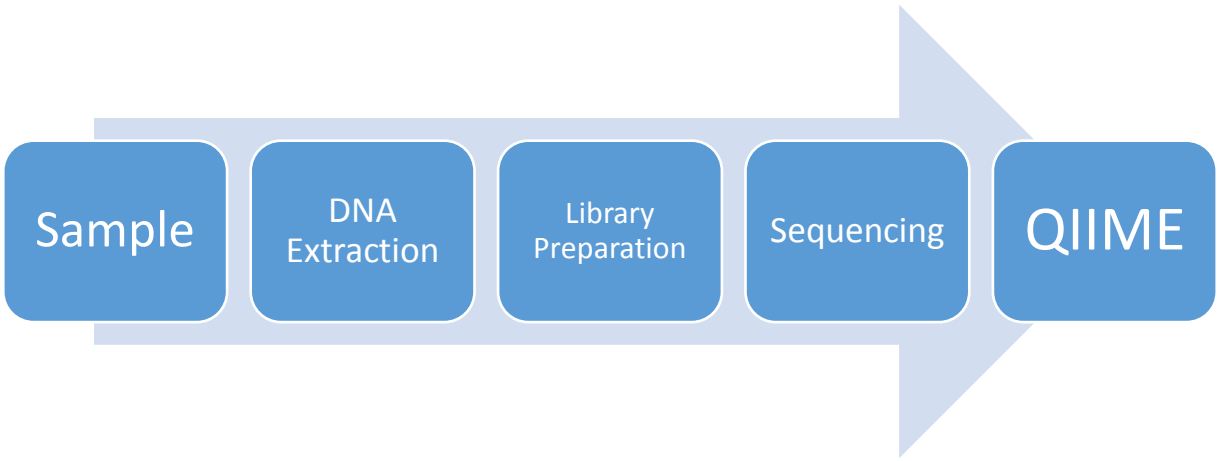

Fig. 2 Microbiome Analysis Workflow

Statistical Methods

Response variables (BW, fecal pH, and fecal VFAs) including relative abundances (RA; >1%) of phyla and genera for each experiment were analyzed with repeated measures ANOVA using the MIXED models procedure of SAS 9.3 (SAS Institution. Inc., Cary, NC) according to Walker [101]. Autoregressive (TYPE = AR(1)) covariance structure was used in this analysis. The model for experiment 1 included the following: treatment, time, and treatment x time. The model for experiment 2 included time. Due to an unexpectedly high variability in fecal pH and VFAs between horses on day 0, d0 values were included as a covariate in the model for experiment 1.

Statistical analysis of microbiota data was carried out using the R package within MacQIIME [102]. Species richness (alpha diversity) was measured by phylogenetic diversity (PD) and Chao1 between sampling days and treatments (restricted and control). Alpha diversity indices were compared using a parametric two sample t-test (compare_alpha_diversity.py).

Community composition or structure, measured by beta diversity, was compared using Principle Coordinates Analysis (PCoA) of the weighted (abundance) and unweighted (composition) UniFrac distances based on the experimental OTU abundance matrix at 97% similarity (EXPT1, figure 17; EXPT2, figure 19). Differences in microbiota stability between treatments and over time was evaluated by the Jaccard index method (beta_diversity.py). Statistical significance of UniFrac distances and Jaccard indices were assessed using the analysis of similarity (ANOSIM; compare_categories.py). ANOSIM compares groups of samples based on the variables stated in the metadata file (figure 31) such as treatment or day. Linear discriminant analysis effect size (LEfSe) as described in Segata et al. was utilized to identify differentially abundant taxa between treatments and over time using (EXPT1, figure 20; EXPT2, figure 21) [103]. LEfSe differs from the SAS analysis of relative abundances by the inclusion of taxa with < 1% abundance. A P-value < 0.05 was considered to be significant; P < 0.10 was considered a trend.

CHAPTER III. RESULTS

Pasture Chemical and Botanical Composition

Mean chemical composition of the pasture for d 0-14 was 28.19 ± 3.36% DM, 9.68 ± 0.66% CP, 65.83 ± 0.77% NDF, and 40.21 ± 0.68% ADF; digestible energy content was 1.99 ± 0.03 Mcal/kg (Mean ± SEM). The mixed grass hay chemical composition used prior to d 0 and in EXPT2 was 91.3% DM, 10.5% CP, 62.6% NDF, 46.3% ADF; digestible energy content was 2.05 Mcal/kg. Pasture and hay Chemical composition values are summarized in table 1. Overall pasture botanical composition was 37% common crabgrass, 30% tall fescue (*Festuca arundinacea* MaxQ, Pennington Seed, Madison, GA), 23% yellow foxtail, 8% weeds (horse nettle, curly dock, etc.), and 2% bare spots.

Body Weight

Experiment 1

Mean BW on d0 was 562 ± 27 kg and 607 ± 27 kg for the CON and RES groups, respectively; there was no difference in BW between the groups on d0 ($P = 0.27$). Body weight was not affected by treatment or time, however, body weight of RES decreased by 19 ± 6.6 kg ($P = 0.01$) from d 0 to d 14 and CON increased ($P < 0.01$) 22.25 ± 6.6 kg due to a significant treatment x time interaction ($P < 0.01$) as shown in figure 3.

Experiment 2

Mean BW for the control group was not significantly different over time ($P = 0.98$).

Hay DMI

Hay DMI prior to the study and during EXPT2 was not significantly correlated with fecal pH, total VFA concentration, or molar VFA proportions. Average daily DMI in kg for these two periods are summarized in table 2.

Fecal pH

Experiment 1

Mean fecal pH was 7.31 ± 0.02 and 7.25 ± 0.02 for CON and RES, respectively. Fecal pH was not significantly affected by time ($P = 0.73$) or treatment x time interaction ($P = 0.12$); however, the CON group tended ($P = 0.08$) to be higher (figure 4). Mean fecal pH for EXPT 1 is summarized in table 3.

Experiment 2

Mean pH was not significantly affected by time (figure 5). Mean fecal pH for EXPT 2 is summarized in table 4.

Fecal VFAs

Experiment 1

Mean total VFA concentration was 55.55 ± 1.40 and 53.41 ± 1.48 mM for CON and RES, respectively. Total VFA concentration was not affected by treatment or treatment x time interaction, but decreased significantly over time ($P = 0.01$) as shown in figure 6. Molar proportions were not significantly affected by treatment or treatment x time interaction; however, acetate, propionate, valerate, isobutyrate, and isovalerate significantly differed over time ($P < 0.05$) as shown in figures 7-10. There was a tendency for fecal butyrate molar proportions to increase over time ($P = 0.07$) and tended ($P = 0.07$) to be higher in the RES group (figure 8). Acetate to propionate (A:P) ratio decreased ($P = 0.05$) over time and tended ($P = 0.09$) to be higher in the CON group (figure 11). EXPT 1 fecal total VFA concentration and molar proportions are summarized in table 3.

Experiment 2

Mean total fecal VFA concentration did not significantly change over time ($P = 0.33$) as shown in figure 12. Molar proportions and acetate to propionate (A: P) ratio were not significantly affected by time (figures 13-17). EXPT 2 fecal total VFA concentration and molar proportions are summarized in table 4.

Fecal Microbiome

The combined data of both experiments initially contained a total of 11,334,930 sequences with a length of 325.17 ± 56.0 bases. A total of 3,720 OTUs were observed from the closed reference. Following clustering, the total sequence count was 1,622,614 with a mean of 50,706 per sample.

Experiment 1

Species richness (alpha diversity), measured by Faith's Phylogenetic Diversity (PD) and Chao1 index, was not affected by restricting the time allowed for grazing (PD: $P = 0.30$; Chao1:

$P = 0.51$) however, there was a significant difference in time for both metrics (d 0 to d 7, $P < 0.01$; d 0 to d 14, $P = 0.0001$). The Principle Coordinates Analysis (PCoA) of the UniFrac distances are shown in figures 23 and 24. Microbiota composition (beta diversity) appeared to be influenced by restriction and abrupt diet change during the 14 d period. The abundance of related organisms as measured by the weighted UniFrac distance was different between treatments ($P = 0.001$) and over time ($P = 0.002$). Similarly, community composition (unweighted UniFrac) was also different between treatments ($P = 0.006$) and over time ($P = 0.02$). These results indicate an effect of restricting time allowed for grazing and abrupt change in diet in the short term on beta diversity. Additionally, microbiota stability assessed by the Jaccard index was different between treatments ($P = 0.01$) and time ($P = 0.01$), supporting the aforementioned results. Linear discriminant analysis effect size (LEfSe) results concur with the findings from the repeated measures analysis whereby restricted horses had a higher abundance of *Fibrobacter*, *Clostridium*, *BF311*, *Phascolarctobacterium*, *Akkermansia*, along with undefined genera; control horses had higher abundances of *Ruminococcus*, *Prevotella*, *CF231*, and undefined genera (figure 27-28).

A total of 23 bacterial phyla were identified with 98.3% represented by 5 predominant taxa (figure 20): Bacteriodetes (47.3%), Firmicutes (31.6%), Fibrobacteres (8.5%), Verrucomicrobia (6.9%), and Spirochaetes (4.0%). Mean Bacteriodetes proportions were significantly affected by time ($P < 0.001$) due to an 13.06% increase ($P < 0.001$) from d 0 to d 7 and 6.28% decrease ($P = 0.008$) from d 7 to d 14 (figure 22); there was a tendency ($P = 0.09$) for a treatment x time interaction. Firmicutes tended ($P = 0.09$) to be in higher proportions in the control group (33.25% vs. 29.96%) and decreased by 9.88% ($P = 0.001$) from d 0 to d 14 (figure 23). Fibrobacteres proportions were not affected by time or treatment x time interaction;

however, proportions were higher in the restricted group (5.50% vs. 11.45%; $P = 0.03$). Relative abundances of Verrucomicrobia and Spirochaetes were not significantly impacted by treatment, time, or treatment x time interaction. Raw relative abundance data are shown in table 5.

A total of 206 genera were identified with 89.8% represented by 11 predominant taxa (table 6). The CON group had higher ($P < 0.05$) counts of *Ruminococcus*, an undefined genus from the Firmicutes phylum (Lachnospiraceae family) and an undefined genus from the Bacteriodetes phylum (Bacteroidales order). While the RES group had higher ($P < 0.05$) counts of *Clostridium* and *Fibrobacter*. Many genera were affected by time and decreased ($P < 0.05$) from d 0 to d 14 (table 6). Genera not impacted by treatment, time, or treatment x time were the following: an undefined genus from the Verrucomicrobia phylum and an undefined genus from the Bacteriodetes phylum (BS11 family), *Treponema,* and *Prevotella.* The relative abundances and respective P-values are summarized in table 6.

Experiment 2

Species richness (alpha diversity) as measured by PD and Chao1 appeared to be unaffected by time; however, tended to decrease ($P = 0.06$) from d 0 to d 14, then increase ($P = 0.07$) from d 14 to d 28. The Principle Coordinates Analysis (PCoA) of the UniFrac distances are depicted in figure 26. Both the relative abundance of related organisms (weighted UniFrac distance) and the community composition (unweighted UniFrac distance) were significantly different over time ($P < 0.05$) and therefore appeared to be impacted by an abrupt change in diet. The stability of the microbiota (Jaccard index) was affected by time ($P = 0.02$). Linear discriminant analysis effect size indicated differential abundances between sampling days (figure 30-31). LEfSe results supports the SAS repeated measures analysis on the relative abundances of genera; the following are a few of the taxa with higher abundances on each sampling day. Day 0:

Firmicutes, Clostridia, Actinobacteria, Gammaproteobacteria, Ruminococcaceae, and *Mogibacterium*. Day 7: *Pseudomonas*. Day 14: *Anaerovorax*. Day 21: *Lactobacillus*, *Lachnospira*, and *Oscillospira*. Day 28: Bacteroidetes and *Dorea*. These results indicate an effect following an abrupt change in diet from hay to pasture (and vice versa) on fecal microbiota composition, stability, and the abundances of taxa.

A total of 23 bacterial phyla were identified with 98.09% represented by 5 predominant taxa (figure 21): Bacteriodetes (47.6%), Firmicutes (34.9%), Verrucomicrobia (7.5%), Fibrobacteres (4.7%), and Spirochaetes (3.3%). The relative abundances of Bacteriodetes and Firmicutes (figure 24) were changed over time ($P < 0.05$) while the remaining predominant phyla (Verrucomicrobia, Fibrobacteres, and Spirochaetes) did not differ. Raw relative abundance data are shown in table 7.

A total of 206 genera were identified with 89.77% represented by 11 predominant taxa (table 8). Only two genera changed over time: an undefined member of the Bacteriodetes phylum (Bacteriodales order) and an undefined member of the Firmicutes phylum (Ruminococcaceae family). The mean relative abundance of the undefined Bacteriodetes genera was affected by time ($P < 0.0001$) due to an increase ($P < 0.001$) from 29.95% (d 0) to 50.42% (d 7). Further, the percentage decreased ($P = 0.005$) to 43.42% by the final day at pasture (d 14). A week following the return to the hay diet the proportion decreased ($P = 0.001$) to 34.67% (d 21) and numerically increased slightly to 36.22% (d 28). The mean relative abundance of the undefined Firmicutes genera was also affected by time ($P = 0.04$) due to a decrease from 13.12% (d 0) to 8.17% (d 14) and increase to 12.8% by d 28. These results indicate an effect of an abrupt change in diet from hay to pasture on the proportions of the two most predominant genera. The remaining genera and respective P-values are summarized in table 8.

CHAPTER IV. DISCUSSION & CONCLUSION

The BW results of EXPT 1 supported previous research whereby restricting time allowed for grazing using grazing muzzles is an effective means of weight control. Scale body weight in EXPT 1 differed between two groups of idle geldings grazing on the same pasture. Horses that were in the restricted group experienced a decrease (19 kg) in scale BW compared to the increase (22 kg) of the continuous grazers as expected from prior research [38,42,43]. Since this occurred over a two week period, the differences in the BW could be attributed to gut fill as all horses were fed ad libitum grass hay for 36 d prior to the study (d 0) [94]. The control horses that were continuously grazing maintain a constant intake of feed from d 0 to d 14 and thus have a consistent gut fill when weighed during sampling. This is supported by the EXPT 2 BW results that were unaffected ($P > 0.98$) by time from d 0 to d 28 and the similarity of the mean intakes pre and post experiment (shown in table 2). The RES horses theoretically should have been restricted to a lower pasture intake and therefore lower gut fill during sampling. However, it is important to note that body measurements such as Body Condition Scores were not taken during this experiment and therefore no definitive conclusion could be made of whether the difference in weight was due to tissue accretion or gut fill. Similar studies noted weight loss over a longer period of time [24,44] meaning the treatment effect may have been greater if the study was carried beyond two weeks. Additionally, activity level of restricted horses has been reported to differ from continuous grazers whereby muzzled horses spend the majority of their time standing as opposed to grazing [45]. Although not measured, this behavior was observed following the application of the muzzles. Activity level may have also been influenced by grazing cell size. The 0.45 ha grazing cells were used in lieu of a larger area to ensure that the horses would be muzzled on time although area available for movement was smaller. Distance traveled is

correlated with BW [44] and therefore limited activity in either group could have impacted BW results. Regardless, the grazing muzzles appeared to attenuate BW over the short term.

Fecal pH has been shown to decline with increasing ratios of starch [104,105] in the diet or in carbohydrate overload models [18,47]. Therefore, we hypothesized that fecal pH would be affected by restriction and there was a tendency ($P = 0.08$) in EXPT 1 for this effect where mean pH was lower in the restricted horses (7.40 ±0.07 and 7.12 ±0.07 for CON and RES, respectively) supporting the hypothesis. These data coincide with a similar muzzle based study by Siciliano and Schmitt where no difference was observed in the fecal pH of restricted and continuously grazing horses over a period of 7 d [106]. Interestingly, this does not appear to be the case when horses are limited by turnout time (via removing them from the grazing area). The amount of time allowed for grazing [24] or the time of day [30] does not appear to affect fecal pH; however, the fecal pH of horses that were restricted was significantly different from their continuously grazing counterparts in the aforementioned studies. The effect on fecal pH seems to depend upon the type of restriction (muzzle versus turnout time) as no effect was found in Siciliano and Schmitt who utilized muzzles as compared to the turnout time restricted studies [24,30]. This may be influenced by gut fill. As mentioned before, the continuous grazers should maintain a consistent intake and therefore a constant gut fill while the muzzled horses theoretically have a lower intake and thus a variable amount of gut fill. Horses restricted by time would not have continuous access to feed unless it's provided in the dry-lot/stall when restricted, while muzzled (open muzzle) horses have limited but access to feed. Dry matter intake rate following restriction may also play a role as horses restricted by time have been reported to have higher rates of intake following the restriction period [24,28]. This may explain why such a difference is seen in fecal pH for studies restricted by turnout time in comparison to those using

grazing muzzles. Results from EXPT 2 support this theory as mean fecal pH for the control group remained steady over time. This disagreed with our hypothesis that fecal pH would decrease while out on pasture since carbohydrate proportions would be higher compared to the hay, mimicking the starch overload models. Moreover, when the diet was switched back to the same hay, we hypothesized an increase in fecal pH. The discrepancies here may be explained by diet composition as the hay used prior to this study and during EXPT 2 was a mature mixed grass hay similar in chemical composition to the pasture (table 1). Lengthening the duration of muzzle experiments or comparing methods of restriction to continuously grazing controls could provide additional insight.

Total fecal VFA concentration decreased by 5.48 ±1.66 mM following diet change from d 0 to d 14 in EXPT 1; however, total concentration was not significantly different between the two groups. There was a tendency for molar proportions to be effected by restriction which is consistent with previous findings. Wycoff et al. found differences in molar proportions (excluding butyrate and isobutyrate) in horses that were restricted by turnout time in comparison to continuous grazers [30]. This was not the case in an earlier study of similar design by Pearson and colleagues, where no differences were found in culture batch VFAs between horses that were turned out for 8 hours in the morning to those turned out in the evening [107]. This discrepancy could be attributed to sample size (n = 12 in Wycoff et al. versus n = 6 in Person et al.) which may also be a factor in the current study which used n = 8 and n = 4 for EXPT 1 and 2, respectively. Further, EXPT 1 was conducted in October 2016 in the Piedmont region of North Carolina during which the transition from warm to cool season grasses was taking place as shown by the botanical composition. Assessed by the step-point method, common crabgrass and yellow foxtail were more prevalent than tall fescue and orchardgrass during this time. The

average temperature during this period was 74º F with a minimal precipitation 1.74 inches, most of which was attributed to two days of heavy rain during hurricane Matthew (October 7-8th). However, temperature was not significantly correlated with fermentation parameters: Total VFA concentration (R = 0.67; *P* = 0.20) and fecal pH (R = -0.02; *P* = 0.96). Interestingly, in EXPT 2 following a diet change from hay to pasture (d 0 to d 7), the total VFA concentration for the control group decreased numerically and continued to do so while on pasture; when returned on the hay diet (d 14 to d 21) total VFA increased numerically close to the original d 0 value. Molar proportions over the 28 day period followed the same pattern and were also not significantly different over time. Experiment 2 results suggest adaptation to the abrupt diet change as the shifts in concentration and molar proportions that occurred following the change appeared to normalize. In a similar manner to fecal pH, close chemical composition of the pasture and hay may have impacted results.

The gut microbiome is known to be naturally highly diverse in humans and horses among other animals [2,108]. Diversity measurements allow for comparison of microbial communities within (alpha) and between (beta) samples. Alpha diversity, or species richness within a sample, was measured by Chao1 index and Faith's Phylogenetic Diversity (PD). Neither of these metrics were different between treatments, meaning restricting time allowed for grazing did not appear to impact species richness. Instead we observed that abruptly changing the diet from hay to pasture had a significant effect as alpha diversity decreased from d 0 to d 14. However, species richness during EXPT 2 showed no difference between sampling days for the control horses which indicates that abrupt diet change may not have had a lasting effect on species richness over the span of 28 days. The abundance of related organisms, beta diversity as measured by unweighted UniFrac distance, was effected by treatment (EXPT 1) and time (EXPT 2) as shifts at both the

phylum and genus level were observed. Furthermore, community composition (weighted UniFrac) was also effected by treatment and time. UniFrac distance is visualized by the principal coordinates analysis which shows clustering of samples by treatment (figure 23-24) and time (figure 25-26). Microbial stability as measured by the Jaccard index also appeared to be affected by treatment and time. These results indicate effects of both pasture restriction using grazing muzzles and abrupt change in diet on fecal microbial diversity. Differences of which can be attributed to observed shifts at both the phylum and genus level.

The 16S rDNA sequencing analysis of the V3 and V4 region resulted an average of 50,706 sequences per sample and the identification of 3,720 operational taxonomic units. Bacterial phyla were in similar proportions in both experiments with the two most predominant phyla being Bacteroidetes and Firmicutes as expected from previous studies [2,11,13-15,71,109,110]. Abundances of phyla were different over time due to a significant time effect (Firmicutes) and a treatment by time interaction (Bacteroidetes). The difference between d 0 and d 7 was the most dramatic in both experiments; Firmicutes decreased by 9.92 ±2.57% and Bacteroidetes increased by 13.06 ±2.01% in EXPT1. During EXPT2 the same dramatic shift occurred as Bacteroidetes increased 17.77 ±2.53% from d 0 to d 7 and Firmicutes decreased by 12.77 ±3.76% from d 0 to d 7 (figure 21). Similar shifts in these two phyla have been observed in fillies undergoing a diet change from a grain diet to pasture [14]. In a similar manner to total VFA concentration, the microbial shifts at the phylum level appear to normalize over time and resemble d 0 values after the control horses were placed back on the hay diet during EXPT2 (figure 22). Abundances of Firmicutes and Bacteroidetes significantly differed over time, but this effect was primarily due to the rapid changes from d 0 to d 7, d 7 to d 14, and d 7 to d 21. Mean phyla abundances between the last two sampling days were not statistically different. Bacteria

within the Bacteroidetes phylum are associated with acetate and propionate production while those classified under Firmicutes are butyrate producers [63,111]. Despite these known functions, molar VFA proportions did not follow the proportion changes of the phyla, attesting to the complexity of the relationship between diet, the microbiota, and fermentation parameters. From the results of the current study, abruptly changing the diet from hay to pasture appears to effect the microbial populations at the phylum level; proportions tend to stabilize following the periods of diet change.

Decreasing diversity is generally associated with disease states or general dysbiosis within the hindgut. In horses, for example, Elzinga and colleagues observed lower diversity within fecal samples of horses diagnosed with EMS compared to their non-EMS counterparts [17]. However, the opposite was reported to occur in the feces of horses diagnosed with laminitis which experienced higher microbial diversity when compared to healthy controls [71]. Further, results from the current study appear align with horses experiencing colitis and Equine Grass Sickness. Costa and colleagues compared the fecal microbiota of horses with and without colitis [6]. Horses without colitis had fecal microbiomes dominated by Firmicutes at 68% with Bacteroidetes at 40% while those with colitis were dominated by Bacteroidetes at 40% and had a decreased abundance of Firmicutes at 30% with a tendency for abundances to be different between groups ($P = 0.086$ and $P = 0.091$ for Firmicutes and Bacteroidetes, respectively) [6]. Changes in these two phyla have also been found in horses with Equine Grass Sickness where abundances of Bacteriodetes were higher and Firmicutes were lower in horses with EGS compared to healthy controls [79]. Experiment 1 findings could indicate microbial disturbances as proportions of Firmicutes tended ($P = 0.09$) to be lower in the restricted group as compared to the control group (33.25% vs. 29.96%). This difference could potentially be explained by their

scale body weight and body condition score. Although not measured in this current study, the average pre-experiment BCS was 7 for both CON and RES, respectively [94]. Based off of body condition scores the majority of these horses could be considered overweight with an average pre-experiment BW of 562.75 kg for CON and 607.50 kg (± 27.69) for RES. Excess BW has been associated with increased risk of developing systemic inflammation, insulin resistance, and laminitis [26]. Firmicutes is found to be higher in obese mice models as well as humans [112,113]. Additionally, Firmicutes proportions increased and Bacteroidetes decreased as body mass index (BMI) increases [113]. Therefore, due to the potential effects of excess body weight, the microbiome of these horses could theoretically reflect those suffering from an inflammatory bowel disease such as colitis. However, the fecal microbiomes of overweight (non-EMS) equids have not been characterized to date. The results of the current study build upon the importance of the two most predominant phyla and the need for further understanding the relationship between their abundance and gut dysbiosis.

At the taxonomic level of genus differences were observed between treatments and over time (Table 6). Most notably, *Ruminococcus* (Firmicutes phylum), an unidentified genus from the Bacteriodetes phylum, and an identified genus from the Lachnospiraceae family were in greater proportions in control horses (0.85 ±0.24%) while *Fibrobacter (Fibrobacteres)* abundances were higher in those that were restricted (5.95 ±2.23%). The results for the control group are consistent with healthy horses (n = 10) on a forage based diet. These horses were reported to have higher proportions of Ruminococcaceae and Lachnospiraceae in a comparison to horses diagnosed with Equine Metabolic Syndrome (EMS) [17]. Stabilization appeared to occur over time as proportions increased to near d 0 values following transition back to the hay diet. For example, *Ruminococcus* and the unidentified genus from the Bacteriodetes phylum

significantly changed over time with a decrease occurring from d 0 to d 7 (5.22 ±1.98%) following the transition from hay to pasture. Additionally, Clostridium (Firmicutes phylum) abundances tended to be higher in the RES group and decreased over time in both groups during EXPT 1, although the differences between days were not significant. The Linear discriminant analysis effect size analysis agreed with the results from repeated measures of both experiments and included taxa that were not analyzed in SAS with abundancies of < 1% (figure 27). Ruminococcaceae, Lachnospiraceae, and Clostridiaceae are all within the Firmicutes phylum and are associated with butyrate production and healthy gut function [111]. In horses, decreasing counts of these taxa have been associated with GI disease states such as colic and colitis [6,13]. The restricted group having significantly lower counts of these taxa shows an effect of grazing muzzle use on fecal microbiota in the short term; this alteration reflects horses suffering from colic and colitis.

Horses have a complex and highly convoluted gastrointestinal tract which begs the question of whether or not feces can be representative of the entire hindgut; a question which is widely debated in the field of microbiome research. Fecal samples have the benefit of being relatively easy to obtain, as ground samples are acceptable if collected properly, and thus serves as a non-invasive and non-lethal sampling method. For this reason, many researchers turn to feces for microbiome analysis. Compartmental studies have varying results but appear to suggest similarities between the distal hindgut and feces. Costa et al. found increasing microbial diversity moving distally along the hindgut; similarities between the cecum and feces were also noted [15]. This is in agreement with an earlier study by Schoster et al., however Dougal et al. found that feces were similar in microbial diversity to the right ventral colon but not comparable to the cecum [11,109]. A 2017 dietary study by Grimm and colleagues showed significant correlations

between microbial composition of the colon and cecum to that of feces following transition from a high fiber to a high starch diet [114]. Additionally, only valerate and propionate VFA proportions in the cecum were correlated with those in the feces. Differences between compartmental studies highlight the need for additional research to build upon our current understanding of the validity of feces in this field. It is imperative to not extrapolate fecal results beyond documented and correlated GI compartments.

Our preliminary study demonstrated that pasture management and abrupt dietary changes can have impacts on microbial fermentation parameters and the fecal microbiome. Furthermore, abruptly changing the diet from hay to pasture had a dynamic effect on both fermentation and microbial diversity. These results support our current understanding regarding the need for a slow transition when changing diets. They also suggest an adaption of the gut microbiota to a new diet, returning the hindgut to normal levels over a period of time. Restricting time allowed for grazing using grazing muzzles resulted in weight loss, however, it did not appear to affect fecal fermentation parameters nor microbial diversity over the short term. A restriction period longer than two weeks is needed to fully understand the effects of wearing a grazing muzzle on these variables. Interestingly, fecal pH and VFAs were affected in other similarly designed studies involving turnout time as the form of restriction. This difference may be due to the limited intake allowed by the open muzzles, or from a greater increased rate of intake observed from said studies. Understanding the effects of using either method of restriction on the hindgut over the long term will allow for implementation of better management practices in regards to horse health and welfare. Additional research is needed to improve our understanding of the relationships between the gut microbiota, diet, and disease. Furthering this knowledge benefits horse owners, managers, veterinarians, and more importantly the horse.

REFERENCES

[1] NRC. 2007. Nutrient Requirements of Horses: Sixth Revised Edition. 6 ed. The National Academies Press, Washington, DC.

[2] Costa, M. C., and J. S. Weese. 2012. The equine intestinal microbiome. Anim Health Res Rev 13: 121-128.

[3] Ursell, L. K., J. L. Metcalf, L. W. Parfrey, and R. Knight. 2012. Defining the Human Microbiome. Nutr Rev 70: S38-44.

[4] Al Jassim, R. A., and F. M. Andrews. 2009. The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic, and stomach ulcers. The Veterinary clinics of North America. Equine practice 25: 199-215.

[5] Kaser, H. T., and Arthur. 2011. Gut microbiome, obesity, and metabolic dysfunction.

[6] Costa, M. C. et al. 2012. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. PloS one 7: e41484.

[7] V. Julliand, P. G. 2016. Horse Species Symposium: The microbiome of the horse hindgut: History and Current Knowledge. Journal of Animal Science 94: 2262-2274.

[8] Wenling, G. et al. 2016. In-depth snapshot of the equine subgingival microbiome. 94: 76–89.

[9] Willing, B. et al. 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. Equine Veterinary Journal 41: 908-914.

[10] Desrousseaux, G. et al. 2012. Effect of collection time on the fermentative activity of microbes in equine faeces. Animal Feed Science and Technology 178: 183-189.

[11] Dougal, K. et al. 2012. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS microbiology ecology 82: 642-652.

[12] Shepherd, M. L., W. S. Swecker, Jr., R. V. Jensen, and M. A. Ponder. 2011. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS microbiology letters 326: 62-68.

[13] Venable, E. B., M. S. Kerley, and R. Raub. 2013. Assessment of equine fecal microbial profiles during and after a colic episode using pyrosequencing. Journal of Equine Veterinary Science 33: 347-348.

[14] Fernandes, K. A. et al. 2014. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PloS one 9: e112846.

[15] Costa, M. C. et al. 2015. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet J 205: 74-80.

[16] Proudman, C. J. et al. 2015. Characterisation of the faecal metabolome and microbiome of Thoroughbred racehorses. Equine Vet J 47: 580-586.

[17] Elzinga, S., J. S. Weese, and A. A. Adams. 2016. Comparison of the Fecal Microbiota in Horses With Equine Metabolic Syndrome and Metabolically Normal Controls Fed a Similar All-Forage Diet. 44: 9–16.

[18] Milinovich, G. J. et al. 2008. Microbial ecology of the equine hindgut during oligofructose-induced laminitis. ISME J 2: 1089-1100.

[19] Shirazi-Beechey, S. P. 2008. Molecular insights into dietary induced colic in the horse. Equine Veterinary Journal 40: 414-421.

[20] Weese, J. S. et al. 2015. Changes in the faecal microbiota of mares precede the development of post partum colic. Journal of Equine Veterinary Science 47: 641-649.

[21] J., L., P. C., B. F., D. A., and S. J. 2015. Understanding Intestinal Microbiota in Equine Grass Sickness: Next Generation Sequencing of Faecal Bacterial DNA. Equine Veterinary Journal 47: 9-9.

[22] Destrez, A., P. Grimm, F. Cezilly, and V. VJulliand. 2015. Changes of the hindgut microbiota due to high-starch diet can be associated with behavioral stress response in horses. 149: 159–164.

[23] Grimm, P., V. Julliand, and C. Philippeau. 2015. 40 Colonic bacterial composition and blood cells in horses undergoing a change of diet. Journal of Equine Veterinary Science 35.

[24] Glunk, E. C., S. E. Pratt-Phillips, and P. D. Siciliano. 2013. Effect of Restricted Pasture Access on Pasture Dry Matter Intake Rate, Dietary Energy Intake, and Fecal pH in Horses. Journal of Equine Veterinary Science 33: 421-426.

[25] Geor, R. J. 2009. Pasture-associated laminitis. The Veterinary clinics of North America. Equine practice 25: 39-50, v-vi.

[26] Geor, R. J., and P. A. Harris. 2013. Equine Applied and Clinical Nutrition Chapter 28 - Obesity. 487-502.

[27] Geor, R. J., and P. A. Harris. 2013. Equine Applied and Clinical Nutrition Chapter 27 - Laminitis. 469-486.

[28] Dowler, L. E., P. Paul D. Siciliano, P. Shannon E. Pratt-Phillips, and P. Matthew Poore. 2012. Determination of Pasture Dry Matter Intake Rates in Different Seasons and Their Application in Grazing Management.

[29] Longland, A. C., C. Barfoot, and P. A. Harris. 2015. 109 The effect of wearing grazing muzzles for 10 hours daily on bodyweight of ponies pastured for 24 hours per day Journal of Equine Veterinary Science 35: 430.

[30] Wycoff, C. L., E. c. Glunk, S. R. Sykes, S. E. Pratt-Phillips, and P. D. Siciliano. 2013. Effect of day-time vs. night-time grazing on hindgut fermentation parameters in fall pasture-fed horses. 33: 338–339.

[31] Treiber, K. H. et al. 2006. Evaluation of genetic and metabolic predispositions and nutritional risk factors for pasture-associated laminitis in ponies. Journal of the American Veterinary Medical Association 228: 1538-1545.

[32] Marr, N. J. M.-G. et al. 2010. Severity and outcome of equine pasture-associated laminitis managed in first opinion practice in the UK.

[33] USDA. 2000. Lameness and laminitis in U.S. Horses. #N318.0400 Fort Collins (CO): USDA, APHIS, VA, CEAH, National Animal Health Monitoring System.

[34] Milinovich, G. J., A. V. Klieve, C. C. Pollitt, and D. J. Trott. 2010. Microbial events in the hindgut during carbohydrate-induced equine laminitis. The Veterinary clinics of North America. Equine practice 26: 79-94.

[35] Undersander, D. 2013. Grass Varieties for Horses Journal of Equine Veterinary Science 33.

[36] GD Potter, F. Arnold, D. Householder, D. Hansen, and K. Brown. 1992. Digestion of starch in the small or large intestine of the equine. Pferdeheikunde 1: 107-111.

[37] Longland, A. C., J. Ince, and P. A. Harris. 2011. Estimation of pasture intake by ponies from liveweight change during six weeks at pasture. 31: 275–276.

[38] Longland, A. C., C. Barfoot, and P. A. Harris. 2011. The effect of wearing a grazing muzzle vs not wearing a grazing muzzle on pasture dry matter intake by ponies. 31: 282–283.

[39] Longland, A. C., and B. M. Byrd. 2006. Pasture Nonstructural Carbohydrates and Equine Laminitis.

[40] Longland, A. C., C. Barfoot, and P. A. Harris. 2016. Effects of Grazing Muzzles on Intakes of Dry Matter and Water-Soluble Carbohydrates by Ponies Grazing Spring, Summer, and Autumn Swards, as well as Autumn Swards of Different Heights. 40: 26–33.

[41] Siciliano, P. D. 2015. Effect of sward height on grazing preference and pasture NSC concentration. Journal of Equine Veterinary Science 35: 435.

[42] Glunk, E. C., C. C. Shaeaffer, M. R. Hathaway, and K. L. Martinson. 2014. Interaction of Grazing Muzzle Use and Grass Species on Forage Intake of Horses. 34: 930–933.

[43] Longland, A. C., C. Barfoot, and P. A. Harris. 2016. Efficacy of Wearing Grazing Muzzles for 10 Hours per Day on Controlling Bodyweight in Pastured Ponies. 45: 22–27.

[44] Gill, J. C., K. E. Lloyd, M. Bowman, P. D. Siciliano, and S. E. Pratt-Phillips. 2017. Relationships Among Digestible Energy Intake, Body Weight, and Body Condition in Mature Idle Horses. 54: 32–36.

[45] Fowler, A. L., J. Parsons, L. Walling, and L. M. Lawrence. 2017. Muzzling affects horse behavior. 52: 101.

[46] Vermorel, M., W. Martin-Rosset, and J. Vernet. 1997. Energy utilization of twelve forages or mixed diets for maintenance by sport horses No. 47. p 157-167.

[47] Davis, M. J. G., R. M. Smith, H. R. Spires, and L. C. 1976. Measurement of Volatile Fatty Acid Production Rates in the Cecum of the Pony.

[48] Crowell-Davis, S. L., and K. A. Houpt. 1985. Coprophagy by foals: Effect of age and possible functions. Equine Veterinary Journal 17: 17-19.

[49] Faubladier, C., S. Sadet-Bourgeteau, C. Philippeau, E. Jacotot, and V. Julliand. 2014. Molecular monitoring of the bacterial community structure in foal feces pre- and post-weaning

[50] Shepherd, M. L., W. S. Swecker, and M. A. Ponder. 2015. Effect of Two Different Commercial DNA Extraction Kits on the Bacterial 16S Ribosomal RNA Gene Denaturing Gradient Gel Electrophoresis Profile of Arabian Gelding Feces. Journal of Equine Veterinary Science 35: 165-169.

[51] Veronique Julliand, A. D. V., Liliane Millet, Gerard Fonty. 1999. Identification of Ruminococcus flavefaciens as the Predominant Cellulolytic Bacterial Species of the Equine Cecum. American Society for Microbiology 65: 3738-3741.

[52] Al Jassim, R. A., P. T. Scott, A. L. Trebbin, D. Trott, and C. C. Pollitt. 2005. The genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract. FEMS microbiology letters 248: 75-81.

[53] Biddle, A. S., S. J. Black, and J. L. Blanchard. 2013. An In Vitro Model of the Horse Gut Microbiome Enables Identification of Lactate-Utilizing Bacteria That Differentially Respond to Starch Induction.

[54] Wilkins, R. I. M., and A. C. 1988. Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract.

[55] Respondek, F., A. G. Goachet, and V. Julliand. 2008. Effects of dietary short-chain fructooligosaccharides on the intestinal microflora of horses subjected to a sudden change in diet. J Anim Sci 86: 316-323.

[56] Sadet-Bourgeteau, S., and V. Julliand. 2012. La diversité de l'écosystème microbien du tractus digestif équin. INRA Prod. Anim. 25: 407-418.

[57] Kamada, N., G. Y. Chen, N. Inohara, and G. Núñez. 2013. Control of Pathogens and Pathobionts by the Gut Microbiota. Nat Immunol 14: 685-690.

[58] Cotter, P. D., R. P. Ross, and C. Hill. 2013. Bacteriocins -- a viable alternative to antibiotics? Nature Reviews. Microbiology.

[59] Edelblum, K. L., and et al. 2017. The microbiome activates CD4 T-cell-mediated immunity to compensate for increased intestinal permeability. Cellular and Molecular Gastroenterology and Hepatology.

[60] Stewart, A. S., S. Pratt-Phillips, and L. M. Gonzalez. 2017. Alterations in Intestinal Permeability: The Role of the "Leaky Gut" in Health and Disease. 52: 10–22.

[61] Rawls, J. M. D. et al. 2017. Microbiota regulate intestinal epithelial gene expression by suppressing the transcription factor Hepatocyte nuclear factor 4 alpha. Genome Research.

[62] Turner, J. R. 2009. Intestinal mucosal barrier function in health and disease. Nature reviews. Immunology 9: 799-809.

[63] Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489: 220-230.

[64] Berg, J. T., B. Chambers, H. Siegel, and A. Biddle. 2017. Equine Microbiome Project: Understanding differences in the horse gut microbiome related to diet. Journal of Equine Veterinary Science 52: 76-95.

[65] APHIS. 2001. Incidence of Colic in U.S. Horses, United States Department of Agriculture.

[66] APHIS. 2017. Equine Mortality in the United States, 2015, Veterinary Services Center for Epidemiology and Animal Health.

[67] Garner, H. E., J. R. Coffman, A. W. Hahn, D. P. Hutcheson, and M. E. Tumbleson. 1975. Equine laminitis of alimentary origin: an experimental model. American journal of veterinary research 36: 441-444.

[68] H. E. Garner, J. N. M., J. H. Johnson, L. Clark, J. F. Amend, L. G. Tritschler, J. R. Coffmann. 1978. Changes in the Caecal Flora associated with the Onset of Laminitis. Equine Veterinary Journal 10: 249-252.

[69] Onishi, J. C. et al. 2012. Intestinal bacterial overgrowth includes potential pathogens in the carbohydrate overload models of equine acute laminitis. Vet Microbiol 159: 354-363.

[70] Moreau, M. M., S. C. Eades, C. R. Reinemeyer, M. N. Fugaro, and J. C. Onishi. 2014. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Veterinary Microbiology 168: 436-441.

[71] Steelman, S., B. Chowdhary, S. Dowd, J. Suchodolski, and J. Janecka. 2012. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Veterinary Medical Research: 231.

[72] Milinovich, G. J. et al. 2007. Fluorescence in situ hybridization analysis of hindgut bacteria associated with the development of equine laminitis. Environmental microbiology 9: 2090-2100.

[73] Eades, S. C. 2010. Overview of current laminitis research. The Veterinary clinics of North America. Equine practice 26: 51-63.

[74] APHIS. 2005. Equine 2005 Part II: Changes in the U.S. Equine Industry, 1998-2005, United States Department of Agriculture.

[75] APHIS. 2001. National Economic Cost of Equine Lameness, Colic, and Equine Protozoal Myeloencephalitis (EPM) in the United States, United States Department of Agriculture.

[76] Weese, J. S. et al. 2015. Changes in the faecal microbiota of mares precede the development of post partum colic. Equine Vet J 47: 641-649.

[77] Rodriguez, C. et al. 2015. Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission. BMC Microbiol 15.

[78] Pirie, R. S., R. C. Jago, and N. P. Hudson. 2014. Equine grass sickness. Equine Vet J 46: 545-553.

[79] Garrett, L. A., R. Brown, and I. R. Poxton. 2002. A comparative study of the intestinal microbiota of healthy horses and those suffering from equine grass sickness. 87: 81–88.

[80] NAHMS. 1998. Baseline reference of 1998 equine health and management, United States Department of Agriculture, Veterinary Services.

[81] Robin, C. A. et al. 2014. Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. Equine Vet J 47: 196-201.

[82] Vrieze, A. et al. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 143: 913-916.e917.

[83] Kulecka, M. et al. 2016. Prolonged transfer of feces from the lean mice modulates gut microbiota in obese mice. Nutrition & metabolism 13: 57.

[84] Handl, S. et al. 2013. Faecal microbiota in lean and obese dogs. FEMS microbiology ecology 84: 332-343.

[85] Frank, N. et al. 2010. Equine Metabolic Syndrome. Journal of Veterinary Internal Medicine 24: 467-475.

[86] Daly, K. et al. 2011. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. The British journal of nutrition 107: 989-995.

[87] Venable, E. B., S. D. Bland, J. L. McPherson, and J. Francis. 2016. Role of the gut microbiota in equine health and disease. Animal Frontiers 6: 43.

[88] Murray, J.-A. M. D., A. Longland, M. Moore-Colyer, C. Dunnett, and A. Longland. 2014. The effect of feeding a low- or high-starch diet on equine faecal parameters. Livestock Science 159: 67-70.

[89] Philippeau, C., S. Sadet-Bourgeteau, M. Varloud, and V. Julliand. 2015. Impact of barley form on equine total tract fibre digestibility and colonic microbiota. Animal 9: 1943-1948.

[90] Dougal, K. et al. 2014. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. PloS one 9: e87424.

[91] de Fombelle, A., V. Julliand, C. Drogoul, and E. Jacotot. 2001. Feeding and microbial disorders in horses: 1-effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. Journal of Equine Veterinary Science 21: 439-445.

[92] Muhonen, S., V. Julliand, J. E. Lindberg, J. Bertilsson, and A. Jansson. 2009. Effects on the equine colon ecosystem of grass silage and haylage diets after an abrupt change from hay. J Anim Sci 87: 2291-2298.

[93] Venable, E. B. et al. 2017. Effects of Feeding Management on the Equine Cecal Microbiota. 49: 113–121.

[94] Moore, J. L., S. E. Pratt-Phillips, and P. D. Siciliano. 2017. Relationships between level of adiposity, voluntary intake, and digestibility in horses. 52: 76–77.

[95] Rayburn, E. B., and S. B. Rayburn. 1998. A Standardized Plate Meter for Estimating Pasture Mass in On-Farm Research Trials. Agronomy Journal.

[96] Galyean, M. L. 1980. Laboratory procedures in Animal Nutrition Research. Bulletin 749B Supelco, Inc. Bellefonte, PA 16823.

[97] Klindworth, A. et al. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies Nucleic Acids Res No. 41. p e1.

[98] Caporaso JG et al. 2010. QIIME pQIIME allows analysis of high-throughput community sequencing data.

[99] Navas-Molina, J. A. et al. 2013. Advancing our understanding of the human microbiome using QIIME. Methods in enzymology 531: 371-444.

[100] DeSantis TZ, H. P., Larsen N, Rojas M, Brodie EL, Keller K, et al. . 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. App Environ Microb 72: 5069-5072.

[101] Walker, G. A. 2001. Common Statistical Methods for Clinical Research with SAS Examples. second ed.

[102] Team, R. C. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

[103] Segata, N. et al. 2011. Metagenomic biomarker discovery and explanation. Genome biology 12: R60.

[104] Whitehouse, C. P. J. D. C. R. J. 2013. Responses in fecal pH from low to high starch intakes in healthy horses. Journal of Equine Veterinary Science 33: 358-359.

[105] Grimm, P., C. Pequegnot, and V. Julliand. 2017. Can fecal parameters easy-to-use in the field reflect hindgut microbiota dysbiosis? . Journal of Equine Veterinary Science 52: 88.

[106] Siciliano, P. D., and S. Schmitt. 2012. Effect of Restricted Grazing on Hindgut pH and Fluid Balance. Journal of Equine Veterinary Science 32: 558–561.

[107] Pearson, A. C., P. D. Siciliano, S. J. McLeod, and V. Fellner. 2011. Effect of turn-out time on pasture NSC intake and hindgut fermentation characteristics. Journal of Equine Veterinary Science 31: 297-298.

[108] Lloyd-Price, J., G. Abu-Ali, and C. Huttenhower. 2016. The healthy human microbiome Genome Med No. 8.

[109] Schoster, A., L. G. Arroyo, H. R. Staempfli, and J. S. Weese. 2013. Comparison of microbial populations in the small intestine, large intestine and feces of healthy horses using terminal restriction fragment length polymorphism. BMC research notes 6: 91.

[110] Dicks, L. M. T., M. Botha, E. Dicks, and M. Botes. 2014. The equine gastro-intestinal tract: An overview of the microbiota, disease and treatment. Livestock Science 160: 69-81.

[111] Louis, P., and H. J. Flint. 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS microbiology letters 294: 1-8.

[112] Ley, R. E. et al. 2005. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America 102: 11070-11075.

[113] Koliada, A. et al. 2017. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC Microbiol 17.

[114] Grimm, P., C. Philippeau, and V. Julliand. 2017. Faecal Parameters as Biomarkers of the Equine Hindgut Microbial Ecosystem Under Dietary Change.

APPENDICES

Table 1.
Nutrient composition of mixed grass hay and pasture.[a]

Item	Hay[b]	Pasture[c]	±SEM[d]
Dry Matter, %	91.3	28.12	3.36
Digestible Energy (Mcal/kg)	2.05	1.99	0.03
Crude Protein, %	10.5	9.68	0.66
Acid detergent fiber, %	46.3	40.21	0.76
Neutral detergent fiber, %	62.6	65.82	0.67
Non-Fiber Carbohydrate, %	18.6	15.06	0.85
Calcium, %	0.67	0.31	0.01
Phosphorous, %	0.14	0.24	0.01
Magnesium, %	0.14	0.28	0.01
Potassium, %	1.51	1.62	0.05
Sodium, %	0.01	0.01	0
Zinc, mg/kg	19	34.5	6.71
Copper, mg/kg	10	6	0
Manganese, mg/kg	39	117.75	11.19

[a] Expressed on a dry matter basis.
[b]Analysis provided by Dairy One Forage Testing Laboratory (Ithaca, NY).
[c]Analysis conducted by North Carolina Department of Agriculture Farm Feed Testing Service (Raleigh, NC).
[d]Standard error of the four pasture samples submitted

Table 2. Average daily DMI of mixed grass hay pre-experiment and experiment 2[a]

	Pre-experiment[b]			EXPT2		
Horse	**Mean**	**±SEM**	**%BW**	**Mean**	**±SEM**	**%BW**
Cruz	13.1	0.6	2.6	13.4	0.4	2.6
Goose	10.7	0.7	2.0	12.9	0.5	2.3
Uno	15.0	0.5	2.5	14.6	0.5	2.4
Vegas	15.2	0.5	2.4	15.4	0.6	2.4

Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 days on pasture. RES horses were allowed to graze freely from 0800 to 1600 and were then muzzled form 1600 to 0800 the following day while CON horses grazed continuously for 24-h per day. CON horses were then housed in stalls and fed mixed grass hay for an additional 14 days.

[a] 24-h intake in kg DM.

[b] Based off of intake data from Moore et al. prior to day 0 [88] .

Table 3. EXPT 1 Effect of restricting time allowed for grazing on fecal volatile fatty acid profiles and pH[a]

	Time[b]				P-values[c]		
Item	**0**	**7**	**14**	**SEM**	**trt**	**time**	**trt x time**
Total VFA, mM					0.38	0.01	0.23
CON	58.96^{a}	56.02^{ac}	51.65^{bc}	1.40			
RES	56.20^{abc}	51.47^{c}	52.54^{c}	1.47			
Acetate					0.11	0.04	0.84
CON	57.11^{a}	55.87^{ab}	54.05^{bc}	0.81			
RES	54.79^{ab}	52.69^{b}	51.89^{c}	0.85			
Butyrate					0.06	0.06	0.50
CON	8.82	9.19	9.63	0.20			
RES	9.59	10.16	10.04	0.20			
Propionate					0.10	0.04	0.57
CON	21.05^{ab}	21.43^{ab}	21.85^{b}	0.31			
RES	21.90^{ab}	22.19^{abc}	23.46^{c}	0.33			
Valerate					0.17	0.04	0.30
CON	4.26^{a}	4.40^{ab}	4.71^{b}	0.09			
RES	4.47^{ab}	4.82^{b}	4.74^{b}	0.09			
A:P					0.09	0.05	0.99
CON	2.76^{a}	2.63^{abc}	2.48^{abc}	0.07			
RES	2.50^{abc}	2.38^{b}	2.23^{c}	0.07			
Fecal pH					0.08	0.72	0.11
CON	7.37	7.23	7.33	0.01			
RES	7.21	7.37	7.15	0.02			

[a]Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 d. The RES horses were allowed to graze freely from 0800 to 1600 h and were then muzzled from 1600 to 0800 h the following day, while CON horses grazed continuously for 24-h per day. The mean values expressed represent the measurements from four individual horses per group.

[b] Means with different superscripts differ within a rows and columns, $P < 0.05$

[c]P-value < 0.05 was considered to be significant; P < 0.10 was considered a trend.

Table 4. EXPT 2 Effect of abrupt change in diet on fecal volatile fatty acid profiles and pH summary

	Time						P-value[b]
Item	**0**	**7**	**14**	**21**	**28**	**SEM**	**time**
Total VFA, mM	60.91	57.97	53.60	55.25	54.72	2.63	0.33
VFA Proportions, mmol/100mol							
Acetate	58.35	57.11	55.29	55.26	54.31	1.33	0.17
Butyrate	8.64	9.01	9.45	9.65	9.61	0.33	0.20
Propionate	20.32	20.71	21.13	21.88	22.20	0.57	0.17
Valerate	4.46	4.68	5.04	5.05	4.93	0.19	0.20
A:P Ratio[c]	2.89	2.77	2.62	2.48	2.44	0.13	0.15
Fecal pH	7.46	7.32	7.42	7.40	7.38	0.07	0.77

[a]Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 d. The RES horses were allowed to graze freely from 0800 to 1600 h and were then muzzled from 1600 to 0800 h the following day, while CON horses grazed continuously for 24-h per day. The mean values expressed represent the measurements from four individual horses per group.

[b]P-value < 0.05 was considered to be significant; P < 0.10 was considered a trend.

Table 5. EXPT 1 Effect of restricted time allowed for grazing on relative abundances of bacterial phyla*

Phylum		Day (RA, %)[a]				P-values[g]		
	trt	0	7	14	SEM	trt	time	trt x time
Bacteroidetes	CON	39.4[a]	57.17[b]	50.52[c]	2.42	0.19	<0.001	0.09
	RES	41.92[ad]	50.27[bce]	44.35[ade]	2.42			
Firmicutes	CON	40.92[a]	28.15[b]	30.67[bc]	2.37	0.09	<0.001	0.52
	RES	35.40[a]	28.32[b]	25.87[bcd]	2.37			
Fibrobacteres	CON	5.95[a]	5.37[a]	5.20[a]	2.56	0.03	0.26	0.20
	RES	9.47[abc]	8.30[ab]	16.60[c]	2.56			
Verrucomicrobia	CON	8.12	4.82	8.35	1.57	0.82	0.22	0.50
	RES	6.95	6.25	7.02	1.57			
Spirochaetes	CON	3.65	3.10	3.75	1.01	0.42	0.94	0.53
	RES	4.05	4.90	4.50	1.01			

Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 days. RES horses were allowed to graze freely from 0800 to 1600 and were then muzzled form 1600 to 0800 the following day while CON horses grazed continuously for 24-h per day. The mean values expressed represent the measurements from four individual horses per group.

*Only phyla >1% were included.

[a] Means with different superscripts differ within a rows and columns, $P < 0.05$

Table 6. EXPT 1 Effect of restricted time allowed for grazing on relative abundances of bacterial phyla

				Day (RA, %)*				P-values[g]		
Phylum	Family	Genus	trt	0	7	14	SEM	trt	time	trt x time
Bacteroidetes	Undefined[h]	Undefined	CON	29.95^{ad}	50.42^{b}	43.42^{ce}	1.73	0.01	<.001	0.01
			RES	32.80^{df}	41.70^{e}	36.25^{f}	1.73			
	BS11	Undefined	CON	1.75	0.37	0.20	1.32	0.19	0.51	0.59
			RES	3.35	3.4	2.8	1.32			
	Prevotellaceae	*Prevotella*	CON	1.37	1.52	1.72	0.36	0.31	0.96	0.15
			RES	1.15	1.05	0.80	0.36			
Firmicutes	Ruminococcaceae	Undefined	CON	13.12^{a}	7.90^{b}	8.17^{ab}	1.64	0.89	0.04	0.64
			RES	11.22	8.65	8.72	1.64			
		Ruminococcus	CON	2.60^{a}	2.50^{ab}	2.37^{b}	0.37	0.01	0.14	0.34
			RES	2.30^{ab}	1.60^{ab}	1.00^{c}	0.37			
	Lachnospiraceae	Undefined	CON	12.30^{a}	8.77^{abc}	9.30^{b}	1.20	0.04	0.01	0.72
			RES	10.10^{ab}	7.15^{bc}	5.72^{c}	1.20			
	Undefined[I]	Undefined	CON	7.42^{a}	5.32^{b}	6.12	0.47	0.12	0.01	0.31
			RES	6.57^{c}	5.72	4.85^{d}	0.47			
	Clostridiaceae	*Clostridium*	CON	0.60	0.45	0.47	0.13	0.08	0.47	0.94
			RES	0.77	0.67	0.60	0.13			
Fibrobacteres	Fibrobacteraceae	Fibrobacter	CON	5.95^{a}	5.37^{a}	5.20^{a}	2.56	0.03	0.26	0.20
			RES	9.47^{ab}	8.30^{a}	16.60^{b}	2.56			
Verrucomicrobia	RFP12	Undefined	CON	7.87	4.77	8.22	1.56	0.69	0.23	0.53
			RES	6.52	5.87	6.65	1.56			
Spirochaetes	Spirochaetaceae	Treponema	CON	3.60	3.10	3.75	0.98	0.42	0.93	0.57
			RES	4.02	4.80	4.47	0.98			

Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 days. RES horses were allowed to graze freely from 0800 to 1600 and were then muzzled form 1600 to 0800 the following day while CON horses grazed continuously for 24-h per day. The mean values expressed represent the measurements from four individual horses per group.

* Means with different superscripts differ within a rows and columns, $P < 0.05$

[g] P-value < 0.05 was considered to be significant; P < 0.10 was considered a trend.

[h] In the order Bacteroidales.

[I] In the order Clostridiales.

Table 7. EXPT 2 Effect of abrupt change in diet over time on mean relative abundances of bacterial phyla*

Phylum	Day (RA, %)[a]						
	0	**7**	**14**	**21**	**28**	**SEM**	**P-value[f]**
Bacteroidetes	39.40[a]	57.17[b]	50.52[c]	45.90[c]	45.22[c]	1.78	<0.0001
Firmicutes	40.92[a]	28.15[b]	30.67[bc]	38.15[ac]	36.60[ac]	2.65	0.02
Fibrobacteres	5.95	5.37	5.20	2.82	4.20	1.35	0.52
Verrucomicrobia	8.12	4.82	8.35	7.70	8.55	1.66	0.51
Spirochaetes	3.65	3.10	3.75	3.07	3.02	0.46	0.69

Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 days on pasture. RES horses were allowed to graze freely from 0800 to 1600 and were then muzzled form 1600 to 0800 the following day while CON horses grazed continuously for 24-h per day. CON horses were then housed in stalls and fed mixed grass hay for an additional 14 days. Mean values represent the measurements from four individual horses.

*Only phyla >1% were included.

[a] Means with different superscripts differ within columns, $P < 0.05$

Table 8. EXPT 2 Effect of abrupt change of diet on relative abundances of bacterial genera

Phylum	Family	Genus	Day (RA, %)*						
			0	7	14	21	28	SEM	P-value[f]
Bacteroidetes	Undefined[g]	Undefined	29.95[a]	50.42[b]	43.42[c]	34.67[d]	36.22[d]	1.54	<0.01
	RF16	Undefined	2.50	1.52	1.52	3.77	2.37	1.38	0.77
	Prevotellaceae	*Prevotella*	2.27	2.15	2.07	2.85	2.70	0.49	0.74
	Paraprevotellaceae	Undefined	0.77	0.72	0.85	1.40	1.05	0.40	0.76
	Ruminococcaceae	Undefined	13.12[a]	7.90[b]	8.17[b]	11.62[abc]	12.80[c]	1.40	0.04
		Ruminococcus	2.60	2.50	2.37	3.07	2.42	0.34	0.61
Firmicutes	Lachnospiraceae	Undefined	12.30	8.77	9.30	10.85	8.90	1.36	0.33
	Undefined[h]	Undefined	7.42	5.32	6.12	6.72	6.50	0.63	0.25
Fibrobacteres	Fibrobacteraceae	Fibrobacter	5.95	5.37	5.20	2.82	4.20	1.35	0.52
Verrucomicrobia	RFP12	Undefined	7.87	4.77	8.22	7.57	8.22	1.70	0.58
Spirochaetes	Spirochaetaceae	*Treponema*	3.60	3.10	3.75	3.07	3.00	0.45	0.69

Eight mature idle geldings with maintenance only requirements were randomly assigned to two (RES and CON) groups for a period of 14 days on pasture. RES horses were allowed to graze freely from 0800 to 1600 and were then muzzled form 1600 to 0800 the following day while CON horses grazed continuously for 24-h per day. CON horses were then housed in stalls and fed mixed grass hay for an additional 14 days. Mean values represent the measurements from four individual horses.

* Means with different superscripts differ within columns, $P < 0.05$

[f] P-value < 0.05 was considered to be significant; P < 0.10 was considered a trend.

[g] In the order Bacteroidales.

[h] In the order Clostridiales.

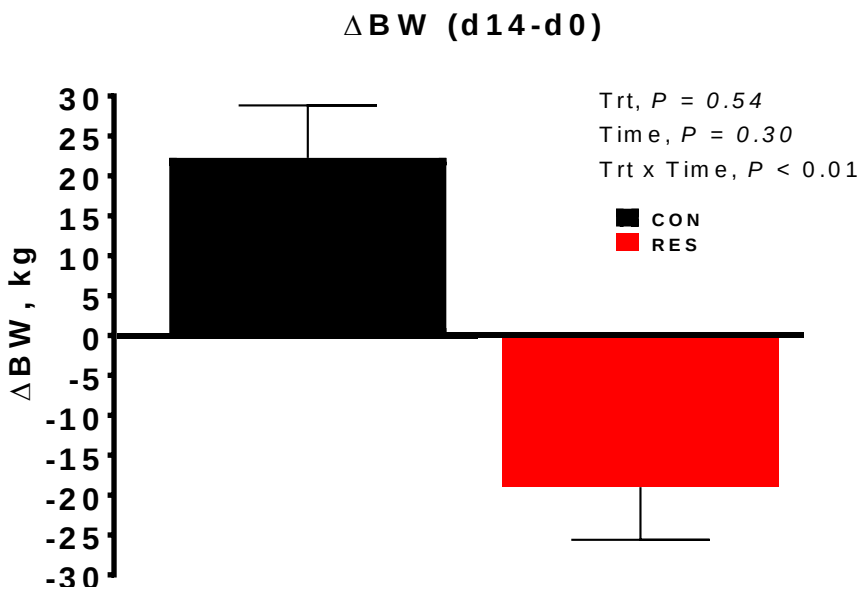

Fig. 3 EXPT1 BW for CON increased (P = 0.005) by 22.25 ± 6.6 kg and *RES decreased (P = 0.01) by 19 ± 6.6 kg.*

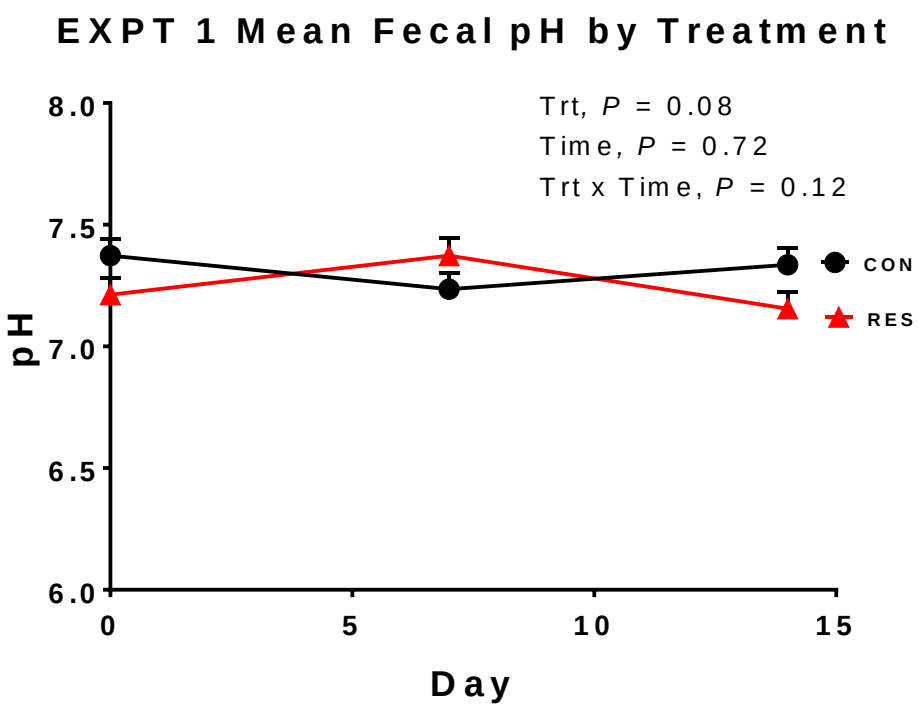

Fig. 4 Mean fecal pH of the control group tended (P = 0.08) to be higher at 7.31 ± 0.01 and 7.24 ± 0.02 for CON and RES, respectively.

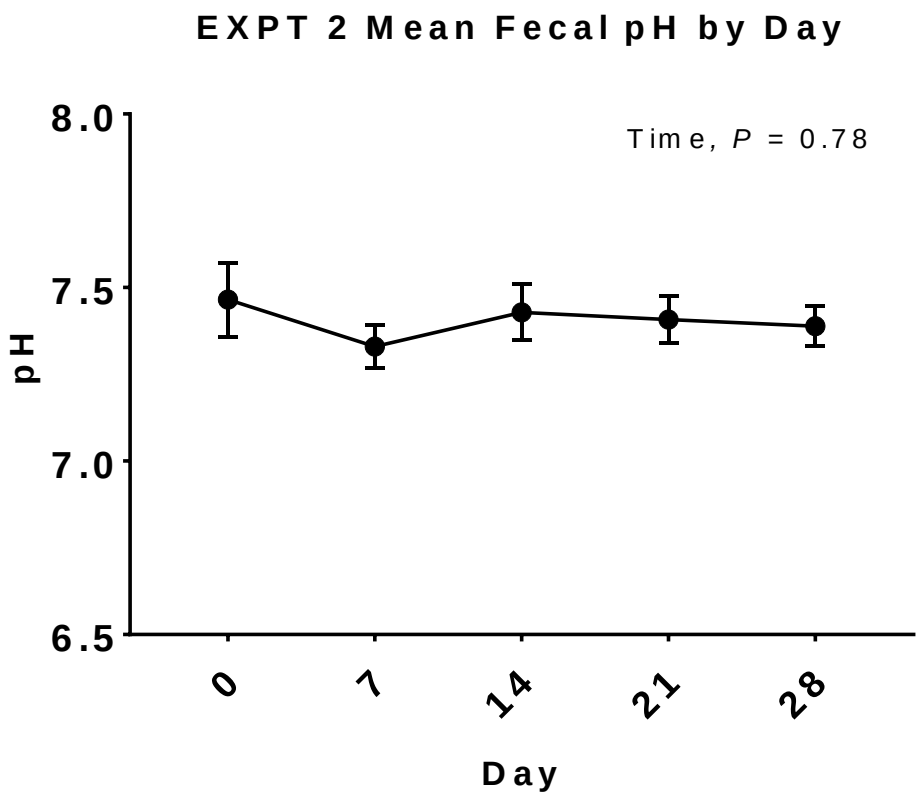

Fig. 5 Mean fecal pH was not significantly different over time.

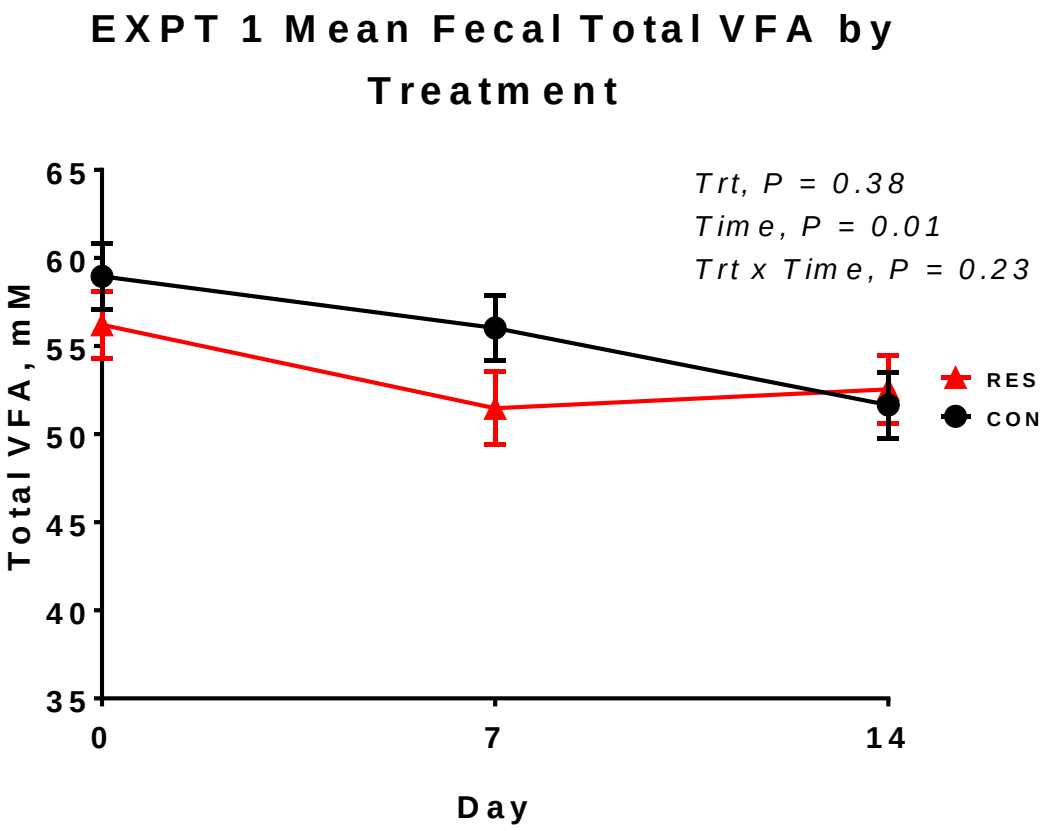

Fig. 6 Mean total VFA concentration decreased (P = 0.007) over time from 57.58 mM (± 1.25) on d 0 to 52.09 mM (± 1.25) on d 14.

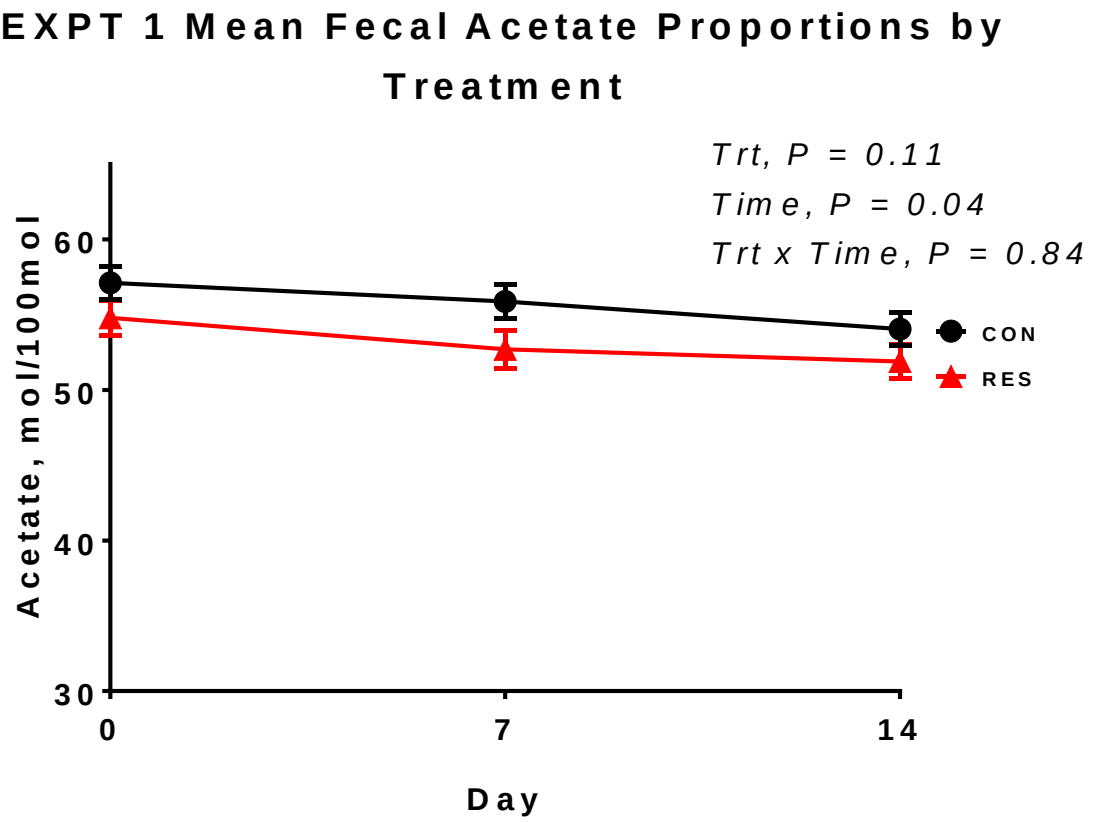

Fig. 7 Mean fecal acetate molar proportions decreased (P = 0.01) from 55.95% ± 0.73 on d 0 to 52.96% ± 0.73 on d 14.

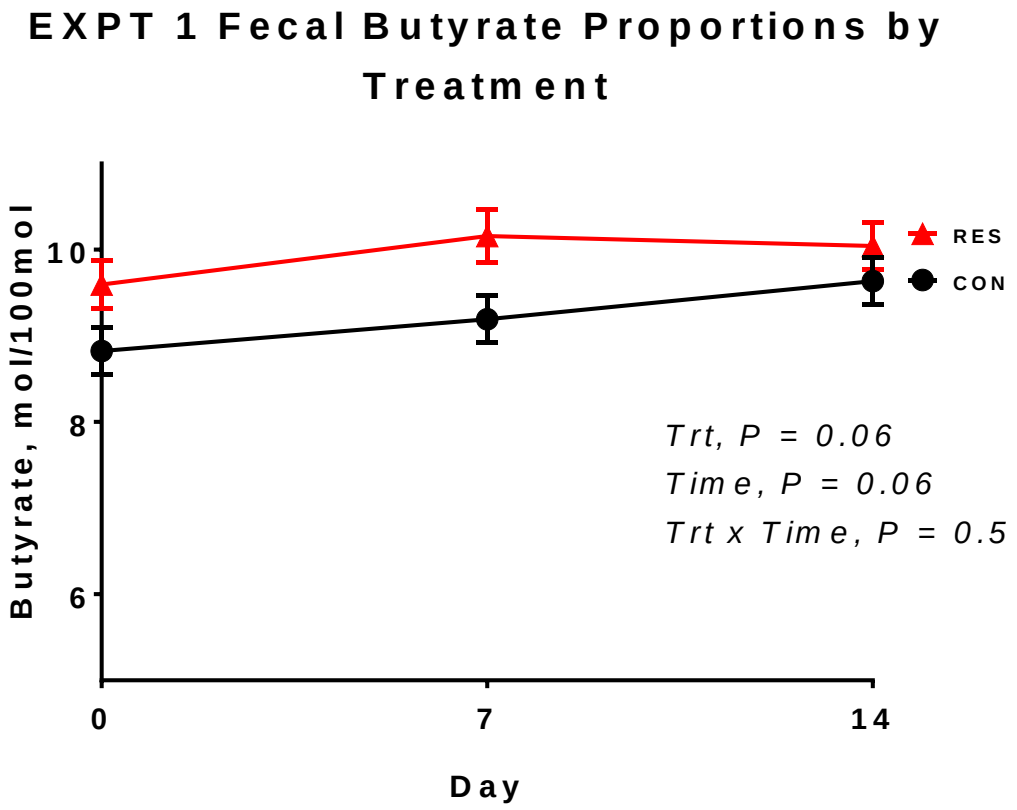

Fig. 8 Mean fecal butyrate molar proportions tended (P = 0.06) to increase from 9.20% ± 0.18 on d 0 to 9.83% ± 0.18 on d 14 and tended (P = 0.06) to be higher in RES horses (9.21% and 9.92% for CON and RES, respectively).

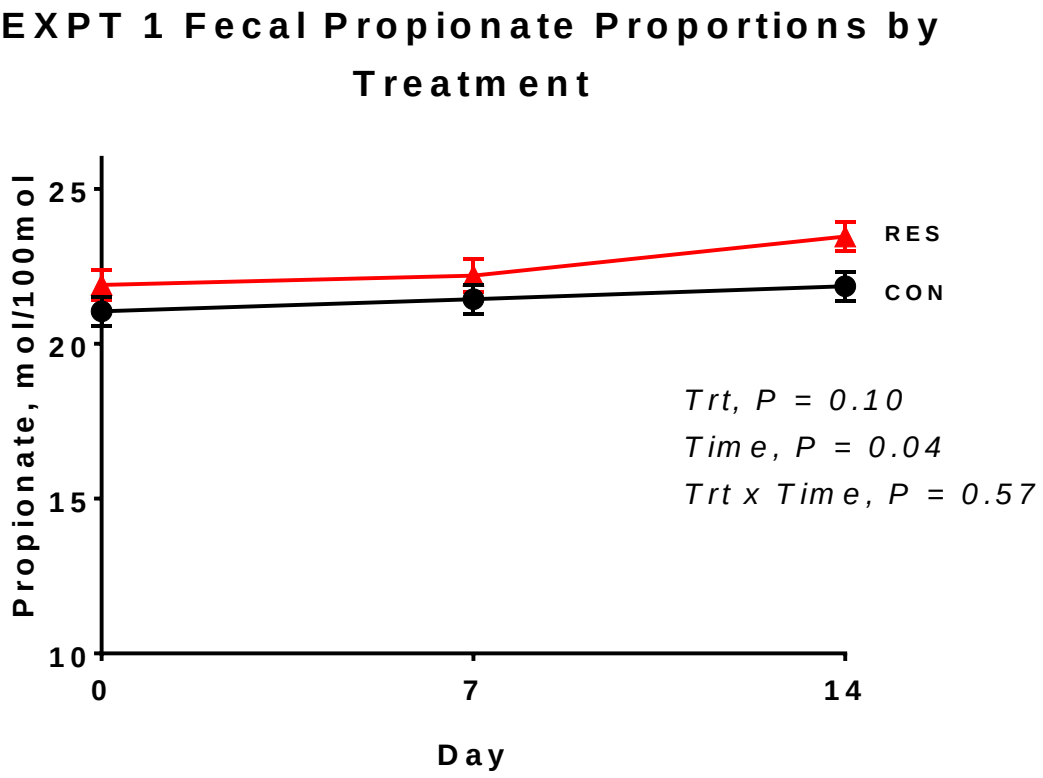

Fig. 9 Mean fecal propionate molar proportions increased (P = 0.01) from 21.47% ± 0.29 on d 0 to 22.65% ± 0.29 on d 14 and tended (P = 0.10) to be higher in RES horses (22.52% and 21.44% for CON and RES, respectively).

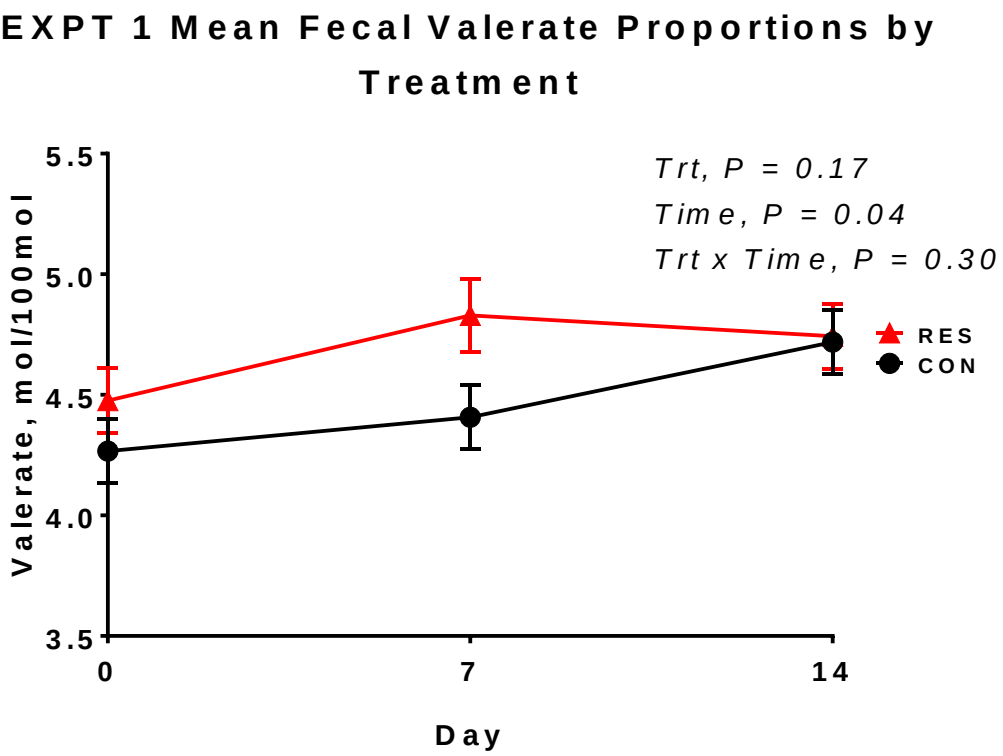

Fig. 10 Mean fecal valerate molar proportions increased (P = 0.01) from 4.37% ± 0.09 on d 0 to 4.73% ± 0.09 on d 14.

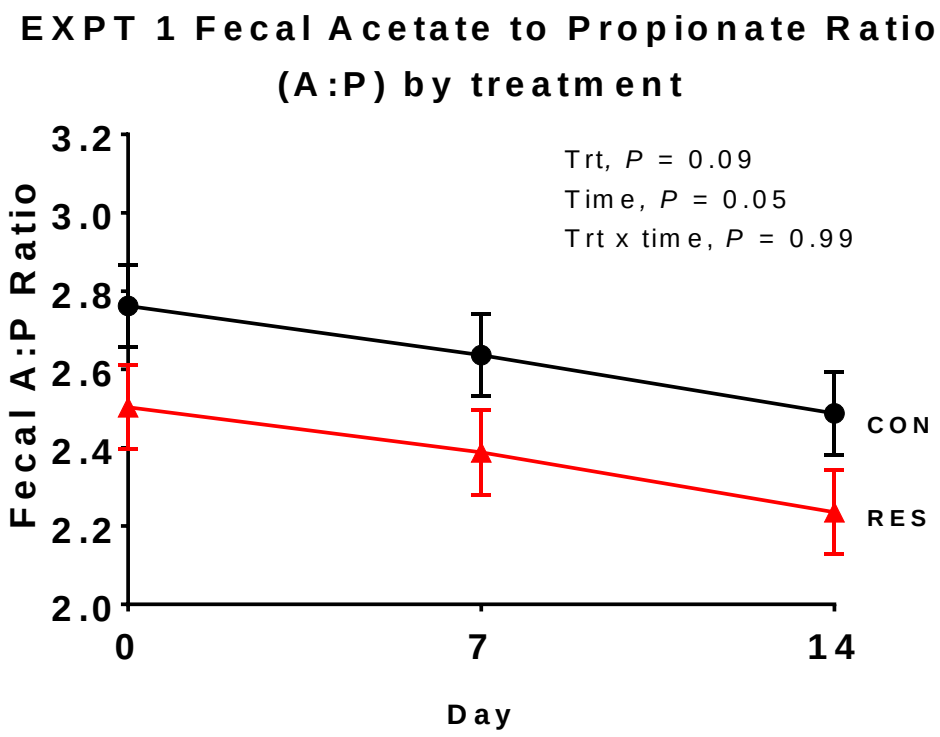

Fig. 11 Mean ratio of acetate to propionate increased (P = 0.05) from 2.63 ± 0.06 on d 0 to 2.36 ± 0.06 on d 14 and tended (P = 0.09) to be higher in the CON group (2.62 and 2.37 for CON and RES, respectively).

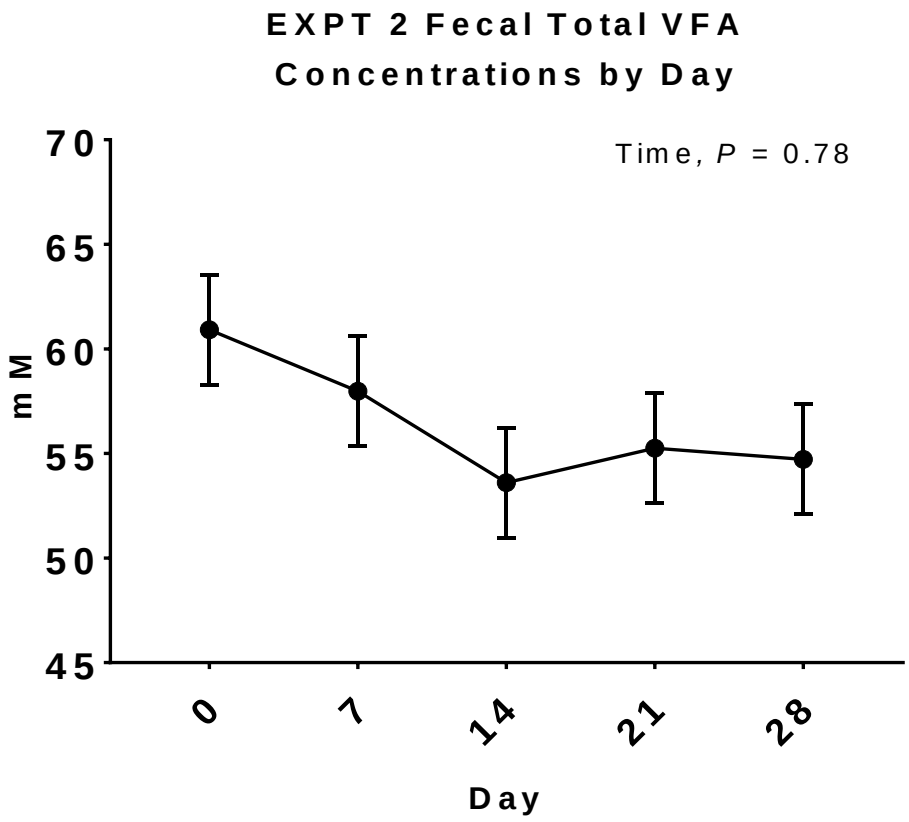

Fig. 12 Mean total VFA concentration tended (P = 0.06) to decrease from 60.91 mM (± 2.63) on d 0 to 53.60 mM (± 2.63) on d 14.

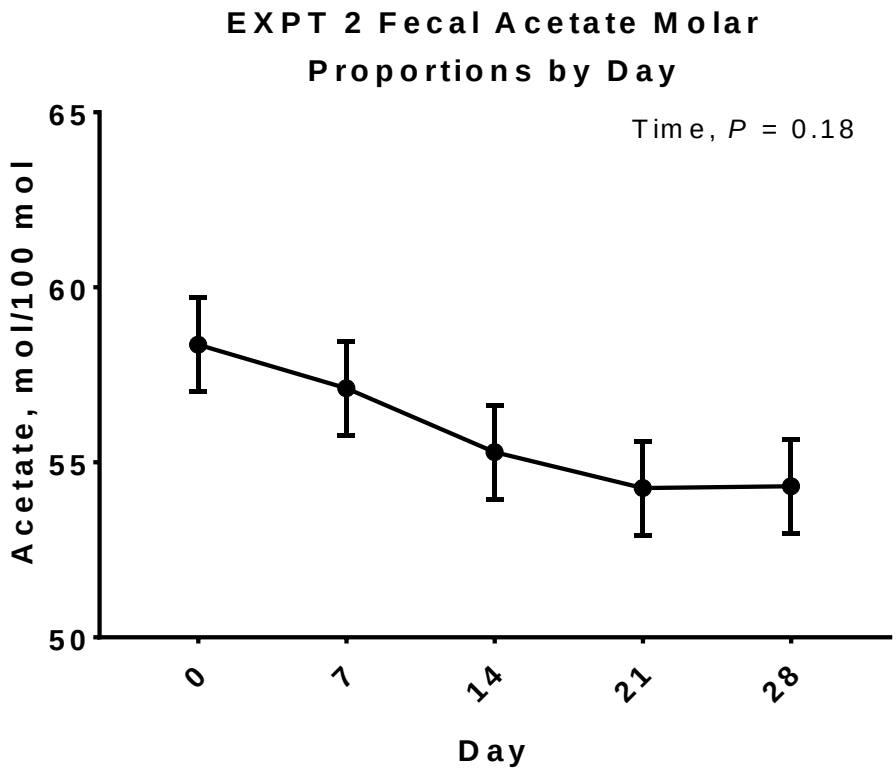

Fig. 13 Mean fecal acetate decreased (P = 0.05) from 58.35% (± 1.33) on d 0 to 54.31% (± 1.33) on d 28.

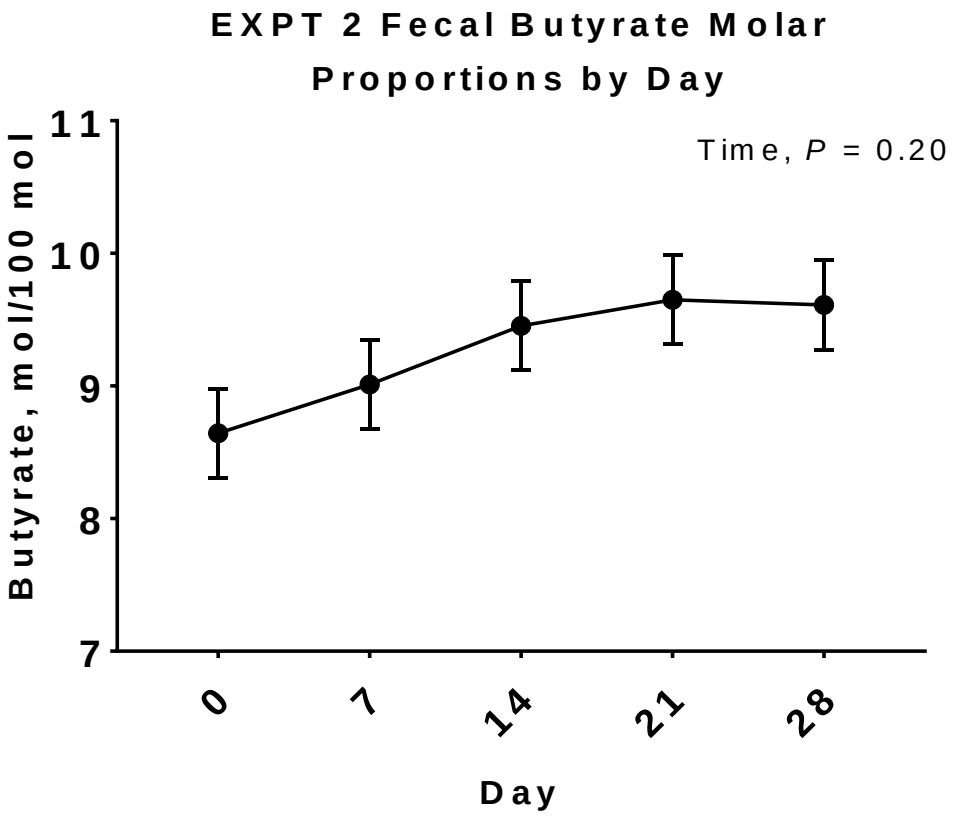

Fig. 14 Mean fecal butyrate increased (P = 0.05) from 8.64% (± 0.33) on d0 to 9.65% (± 0.33) on d 21.

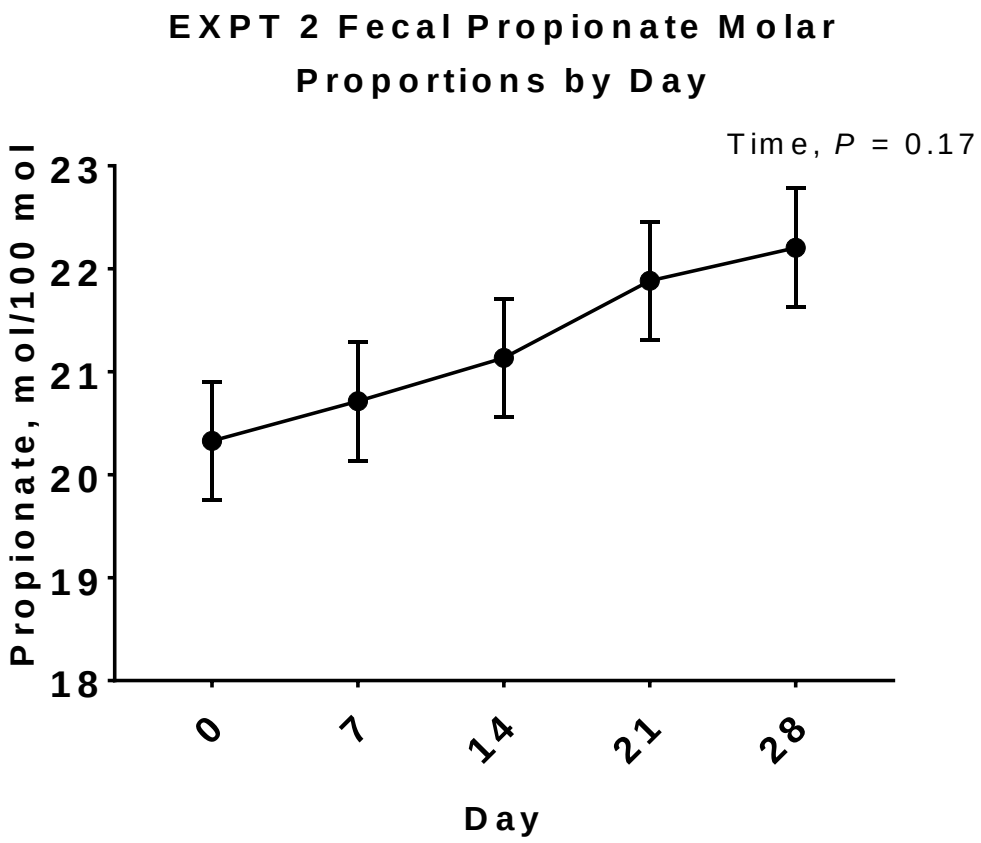

Fig. 15 Mean fecal propionate increased (P = 0.03) from 20.32% ± 0.57 on d 0 to 22.20% ± 0.57 on d 28.

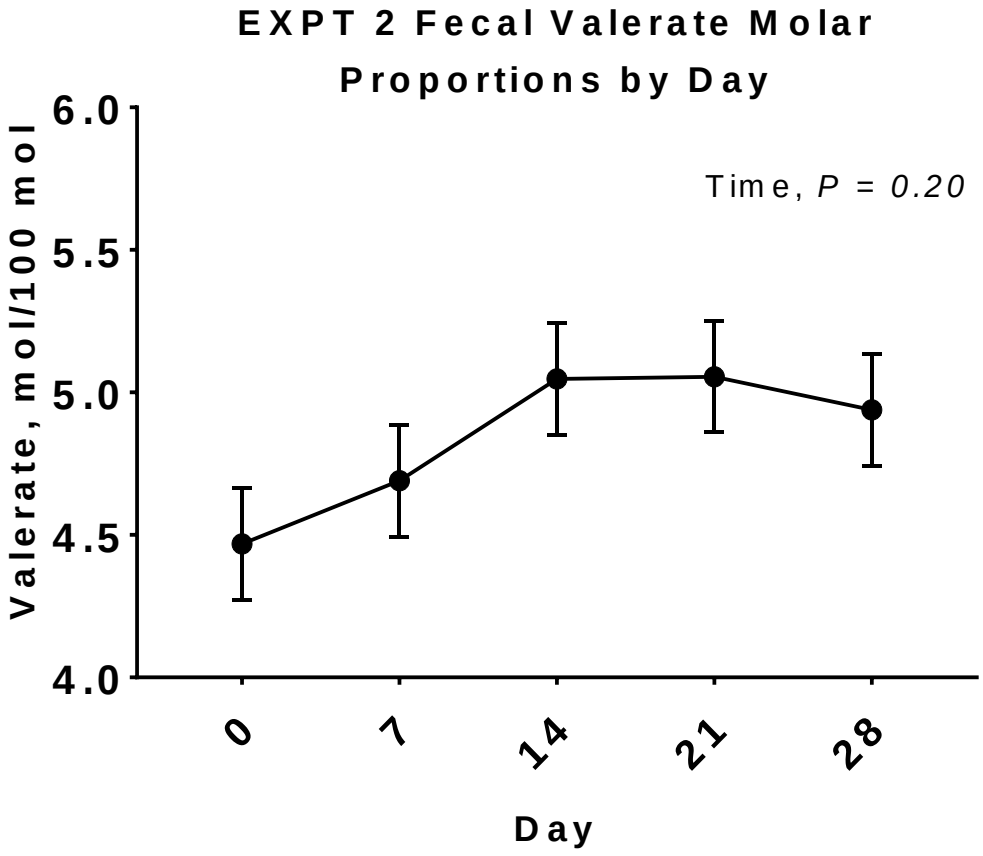

Fig. 16 Mean fecal valerate increased (P = 0.05) from 4.46% (± 0.19) on d 0 to 5.05% (± 0.19) on d 21.

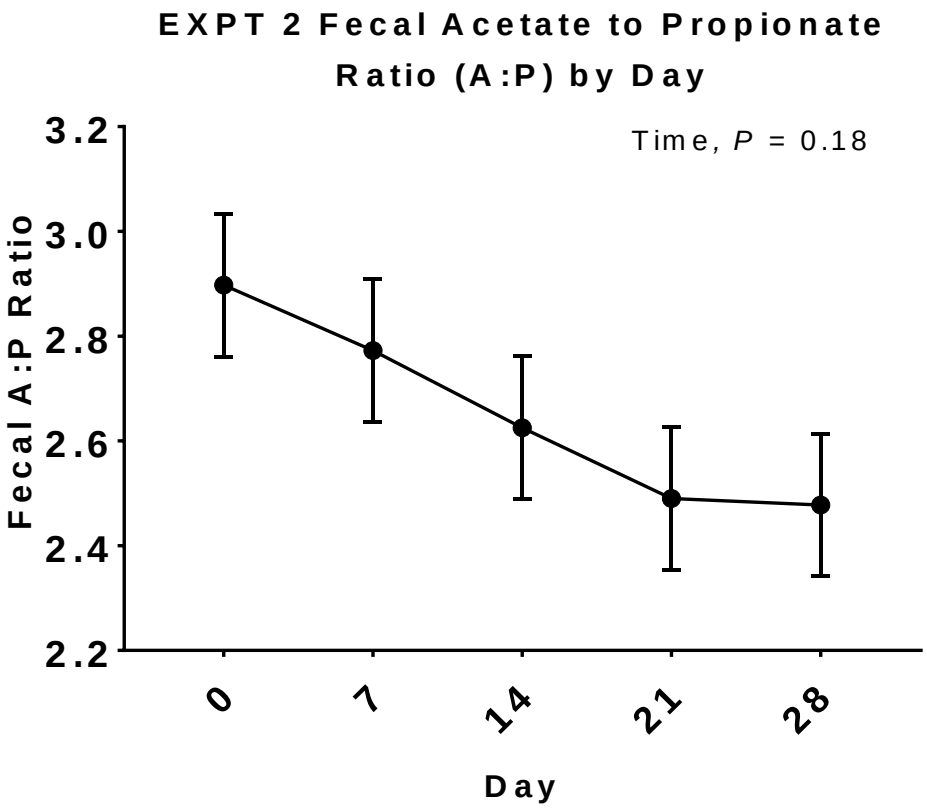

Fig. 17 Mean ratio of acetate to propionate decreased (P = 0.03) from 2.89 (± 0.13) on d 0 to 2.44 (± 0.13) on d 28.

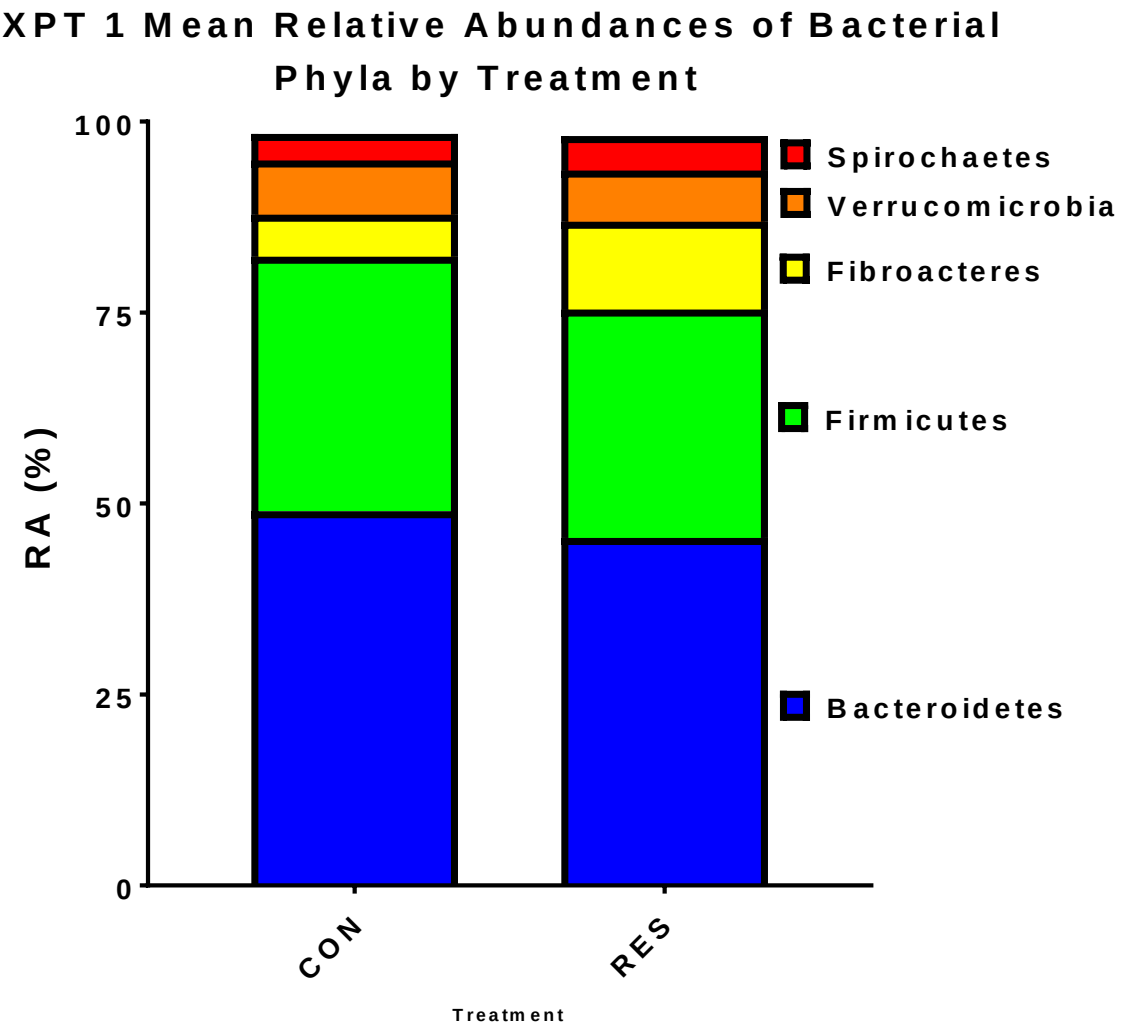

Fig. 18 Relative abundances of Firmicutes tended (P = 0.09) to be higher in CON group (33.25% and 29.86% (± 1.21) for CON and RES, respectively). Fibrobacteres phylum was higher (P < 0.001) in the RES group (5.50% and 11.45% for CON and RES, respectively.

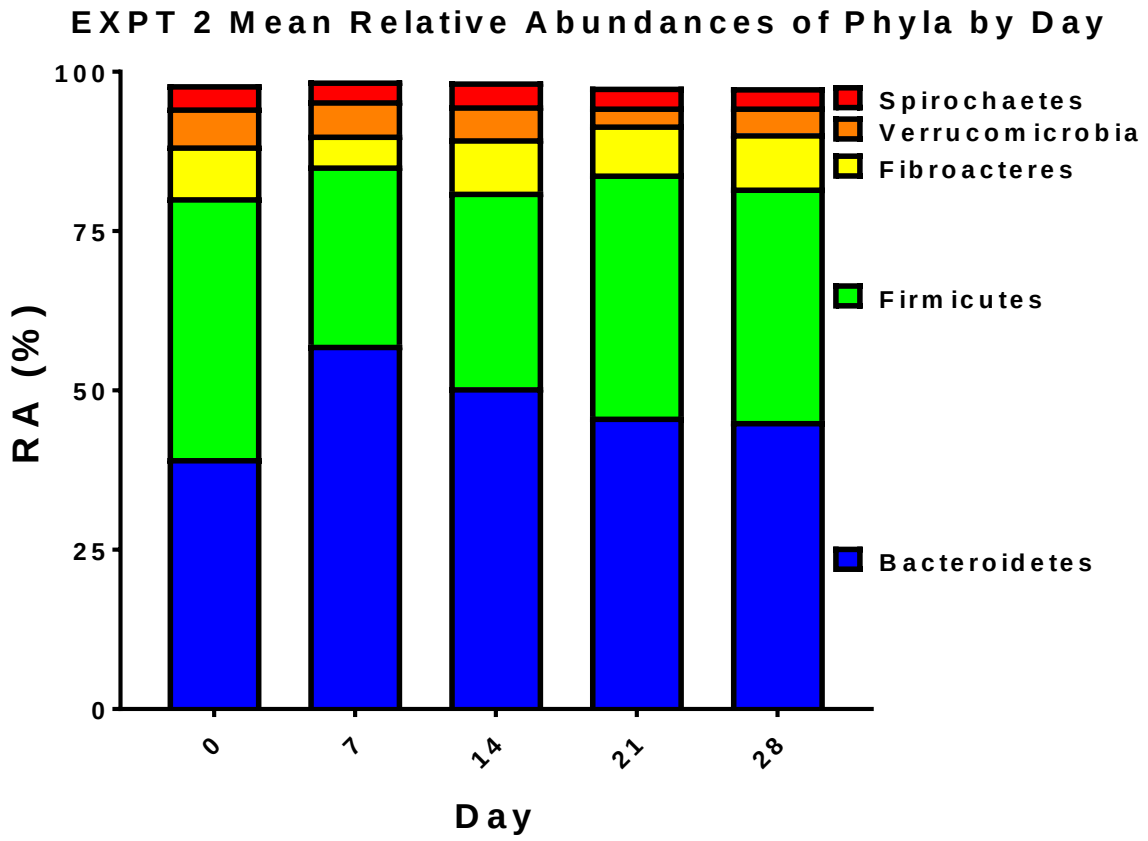

Fig. 19 Relative abundances of Bacteroidetes increased (P < 0.0001) from 39.40% (± 1.78) on d 0 to 57.17% (± 1.78) on d 7 and decreased (P = 0.0005) to 50.52% (± 1.78) on d 14. RA of Firmicutes decreased (P = 0.004) from 40.92% (± 2.65) on d 0 to 28.15% (± 2.65) on d 7 then increased (P = 0.01) to 38.15% (± 2.65) on d 21.

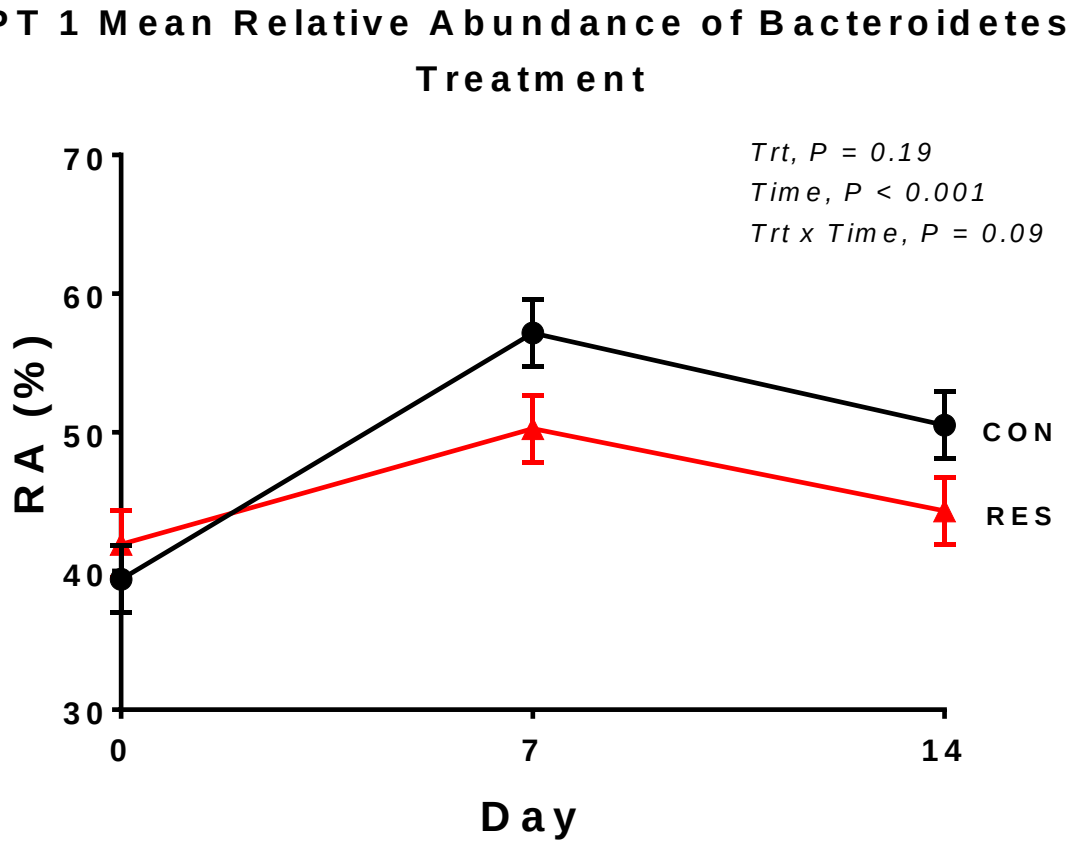

Fig. 20 Relative abundance of Bacteroidetes increased (P < 0.0001) from 40.66% on d 0 to 53.72% (± 1.71) on d 7, then decreased (P = 0.008) to 47.43% (± 1.71) on d 14.

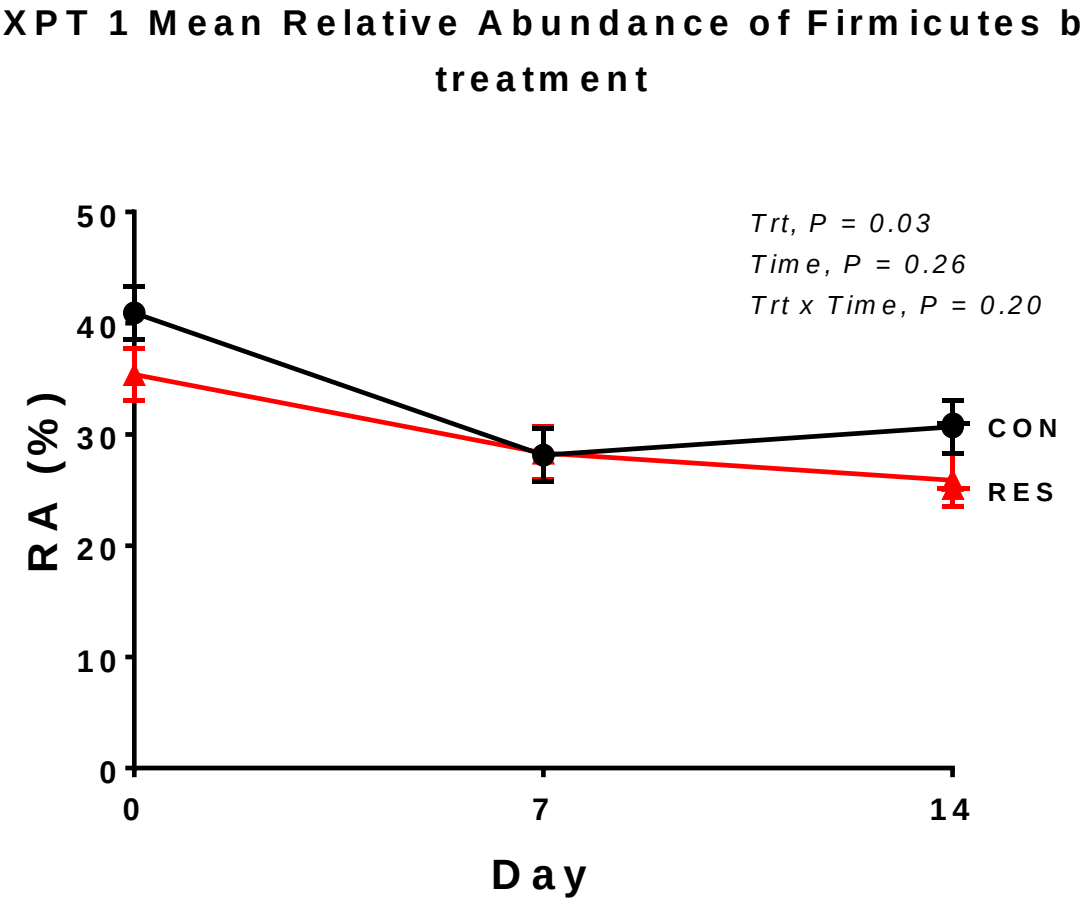

Fig. 21 Relative abundance of Firmicutes decreased from 38.16% (1.68) on d 0 to 28.27% (1.68) on d 14; CON group tended (P = 0.09) to be higher (33.25% and 29.86% for CON and RES, respectively).

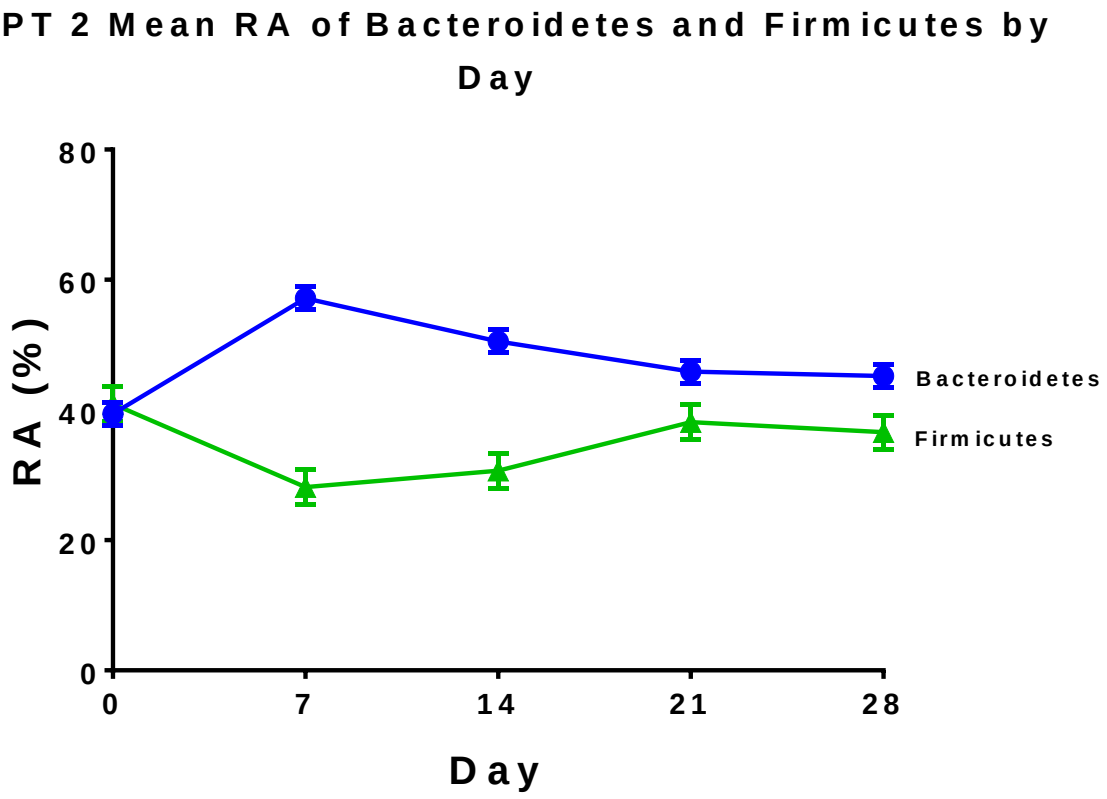

Fig. 22 Relative abundances of Bacteroidetes increased from 39.40% (± 1.78) on d 0 to 57.17% (± 1.78) on d 7, then decreased (P = 0.0003) to 45.22% on d 28 (± 1.78). RA of Firmicutes decreased (P = 0.004) from 40.92% (± 2.65) on d 0 to 28.15% (± 2.65) on d 7, then increased (P = 0.01) to 38.15% (± 2.65) on d 21.

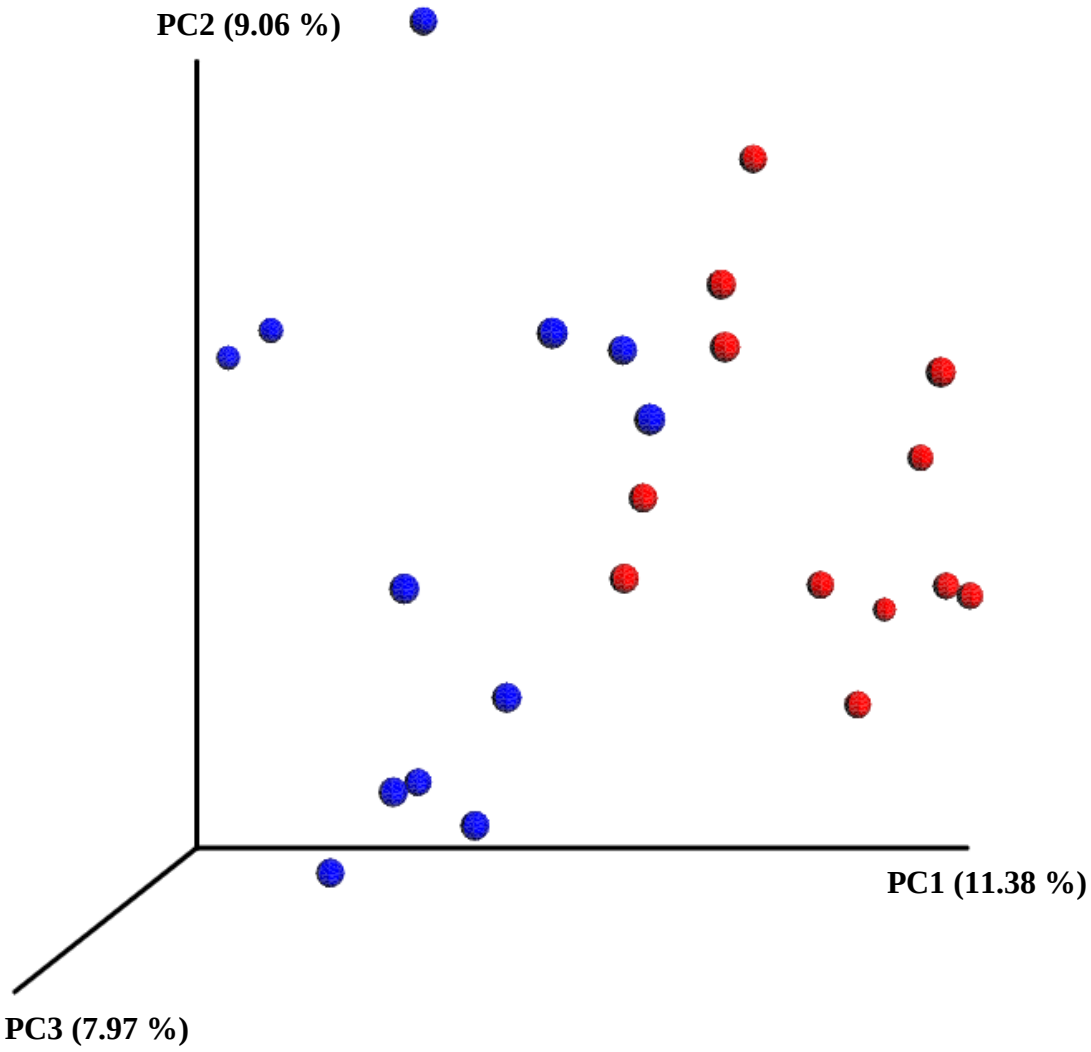

Fig. 23 EXPT 1 Principal Coordinate Analysis Plot of the UniFrac unweighted distance (community composition) as a measurement of beta diversity. Each point represents the microbiome of a single sample from CON (blue) and RES (red) horses.

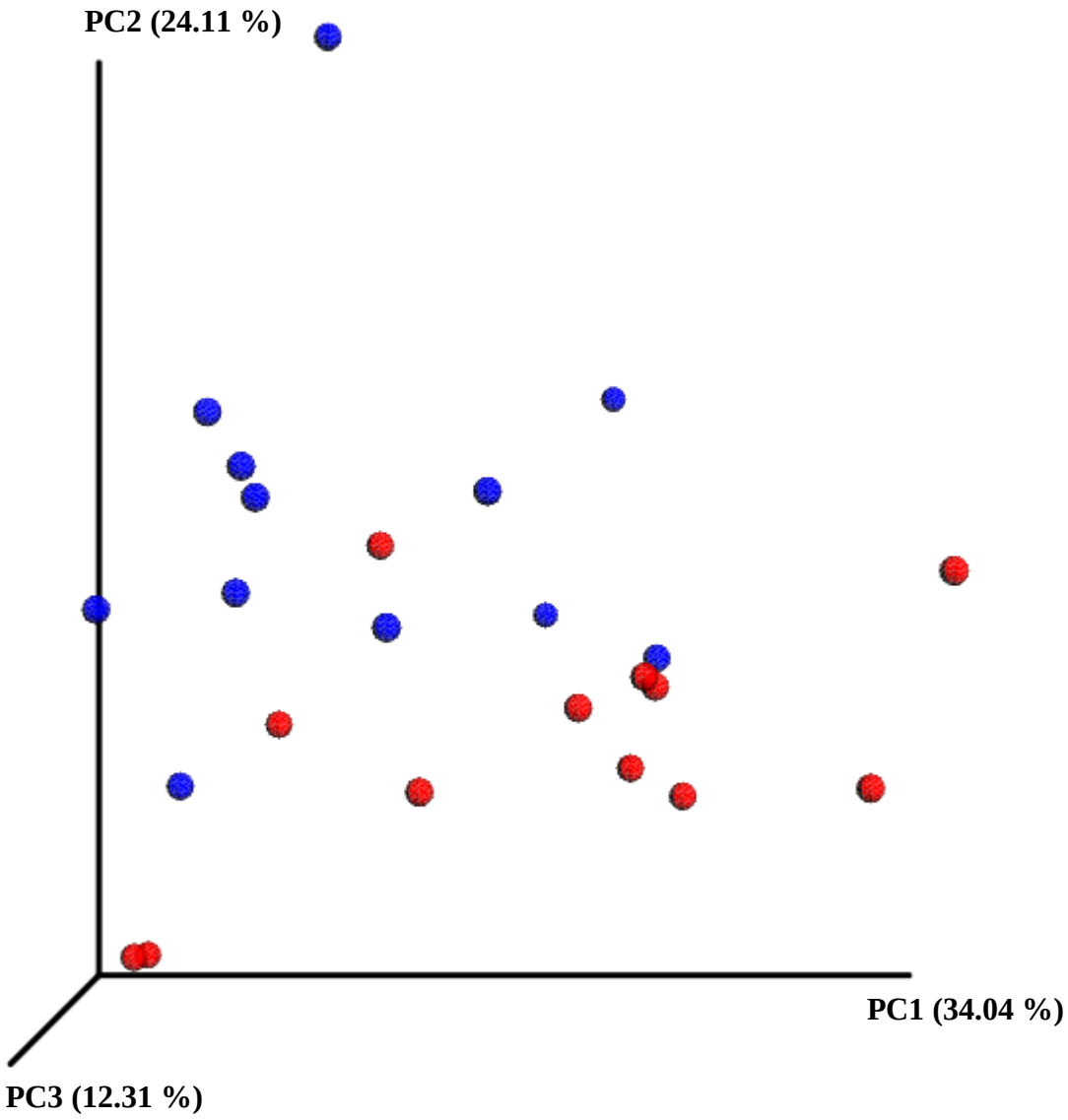

Fig. 24 EXPT 1 Principal Coordinate Analysis Plot of the UniFrac weighted distance (abundance of related organisms) as a measurement of beta diversity. Each point represents the microbiome of a single sample from CON (blue) and RES (red) horses.

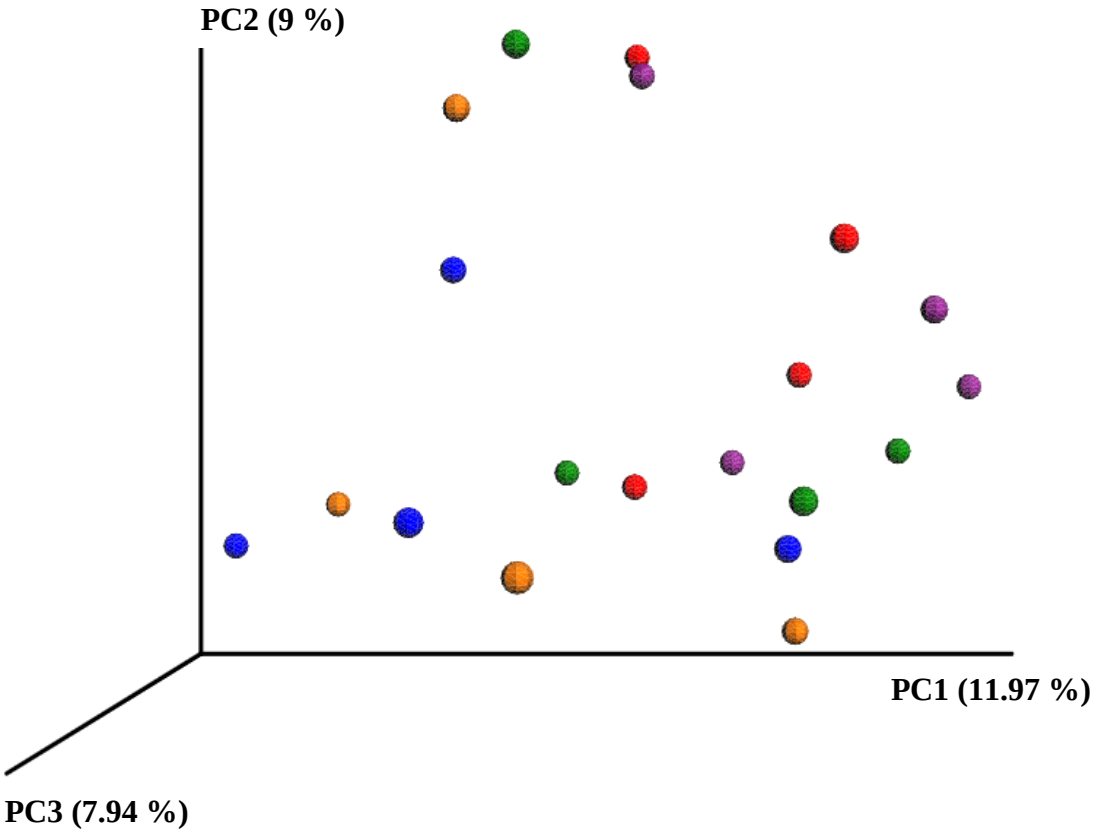

Fig. 25 EXPT 2 Principal Coordinate Analysis Plot of the UniFrac unweighted distance (community composition) as a measurement of beta diversity. Each point represents the microbiome of a single sample at each time point: d 0 (Red), d 7 (Blue), d 14 (Yellow), d 21 (Green), and d 28 (Purple).

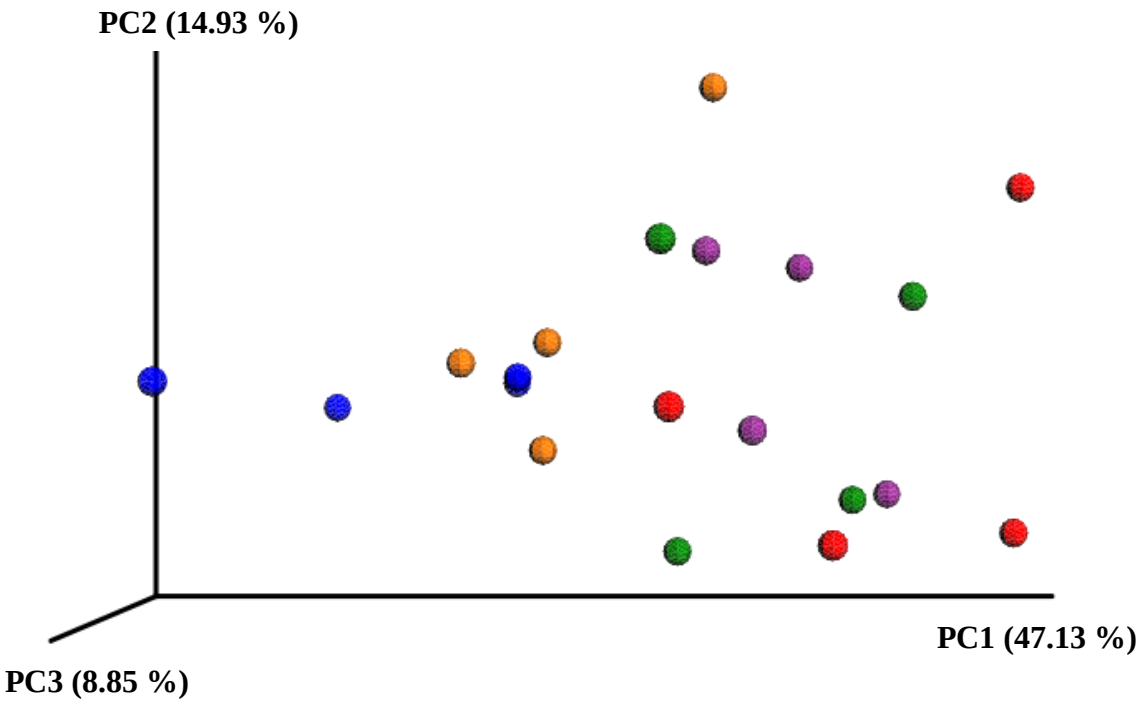

Fig. 26 EXPT 2 Principal Coordinate Analysis Plot of the UniFrac weighted distance (abundance of related organisms) as a measurement of beta diversity. Each point represents the microbiome of a single sample at each time point: d 0 (Red), d 7 (Blue), d 14 (Yellow), d 21 (Green), and d 28 (Purple).

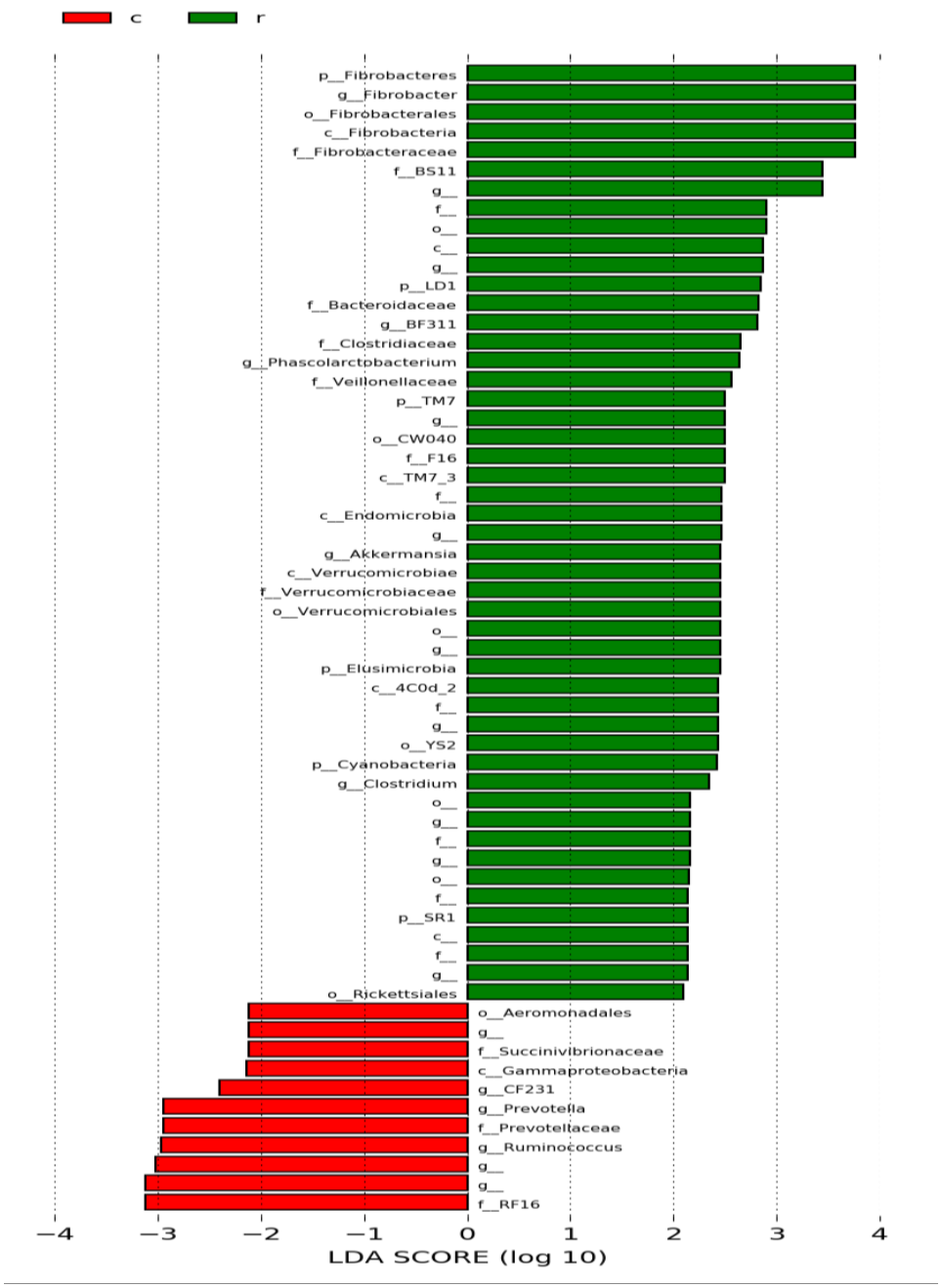

Fig. 27 EXPT 1 Linear Discriminant Analysis (LEfSe) Plot by Treatment. Effect size (LDA) is represented by bars. Absolute values are used for LDA scores. Taxa listed within each group are significantly higher in abundance compared to the other group.

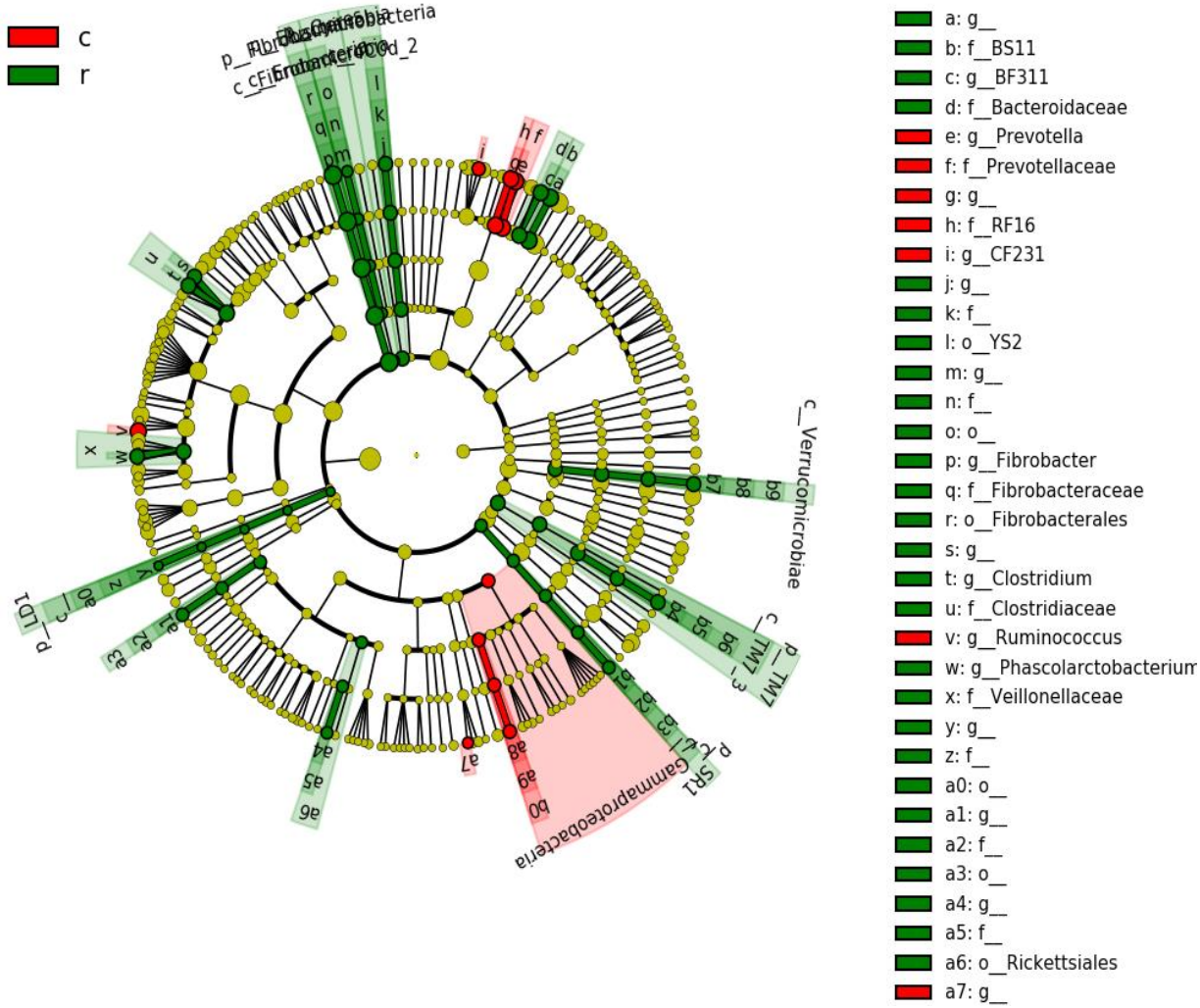

Fig. 28 EXPT 1 Linear Discriminant Analysis (LEfSe) Cladogram by Treatment. This graph visually represents the phylogenetic relationships between taxa within each group.

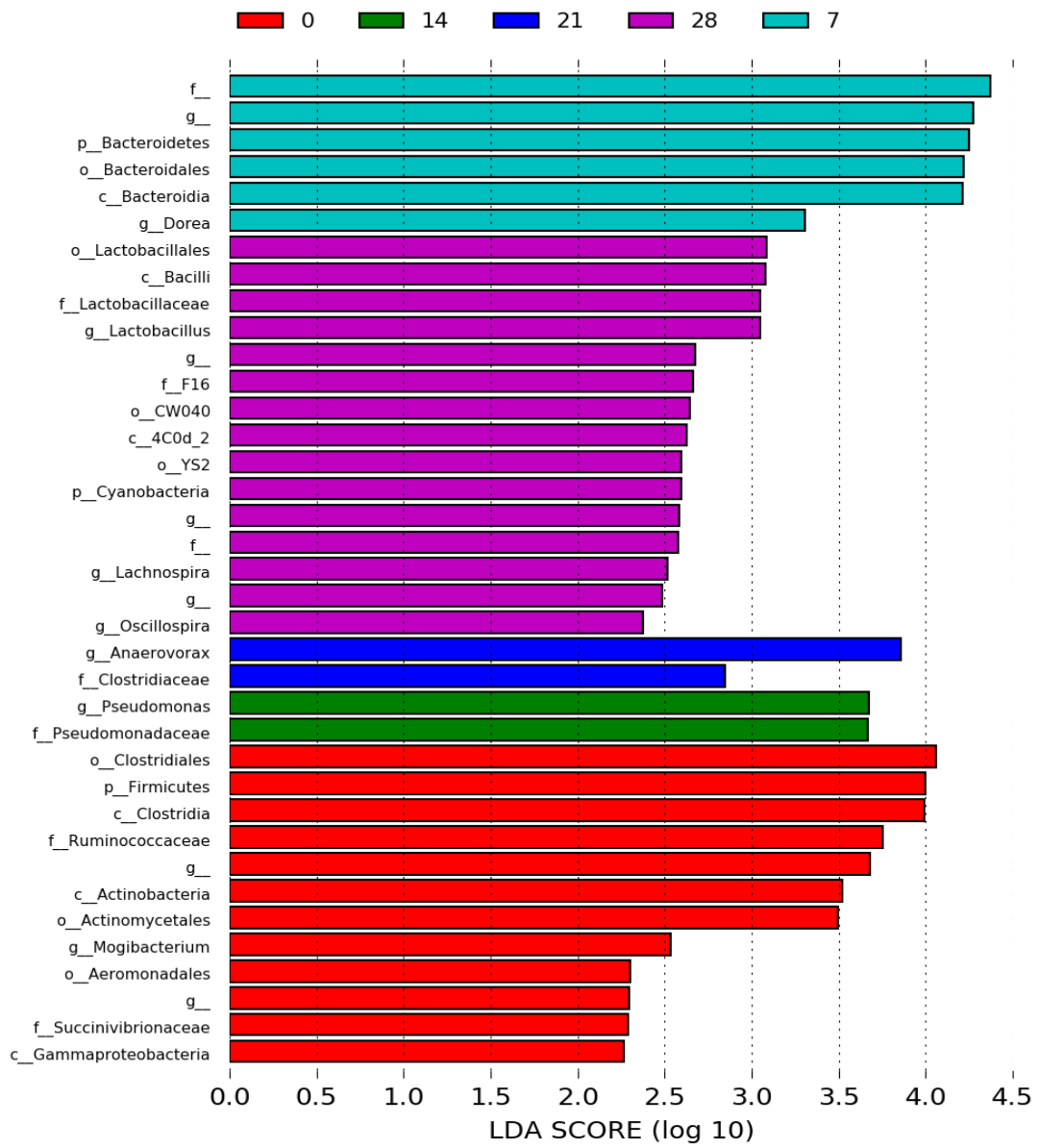

Fig. 29 EXPT 2 Linear Discriminant Analysis (LEfSe) Plot by Day. Taxa listed within each day significantly higher in abundance compared to the other days. Order is determined alphabetically. Effect size (LDA) is represented by bars.

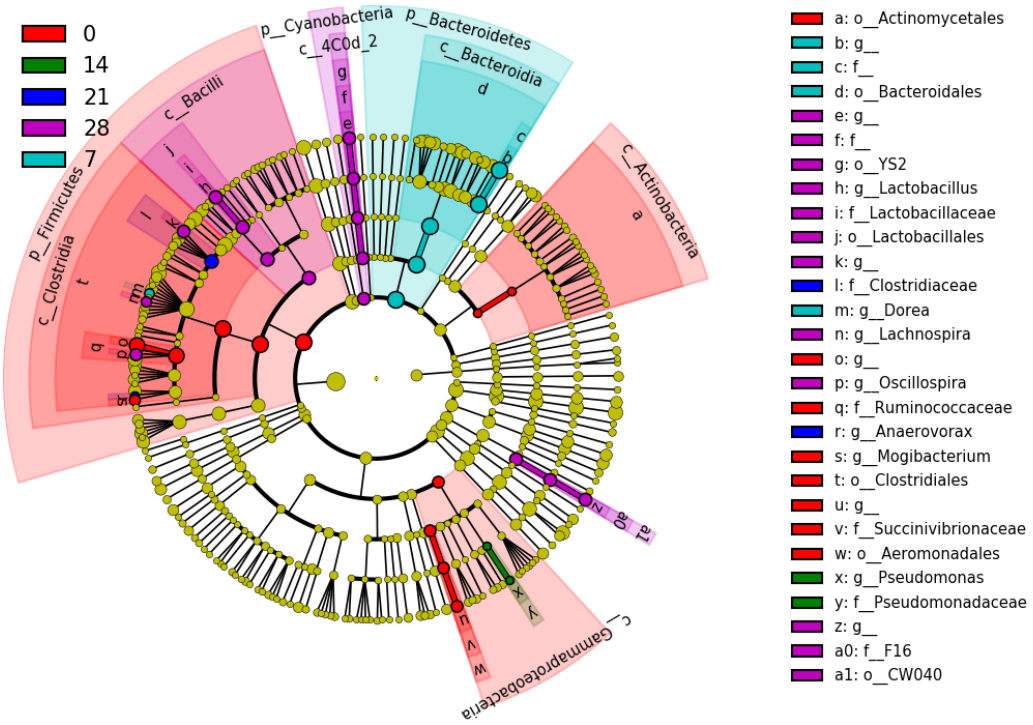

Fig. 30 EXPT 2 Linear Discriminant Analysis (LEfSe) Cladogram by Day. This graph visually represents the phylogenetic relationships between taxa within each group.

#SampleID	BarcodeSequence	LinkerPrimerSequence	Horse	Day	Treatment	Subject	Description
S1	AGCTGACTAGTC	GTGCCAGCMGCCGCGGTAA	Apollo	0	r	1	horse
S2	ATGGCAGCTCTA	GTGCCAGCMGCCGCGGTAA	Apollo	7	r	1	horse
S3	ACACACTATGGC	GTGCCAGCMGCCGCGGTAA	Apollo	14	r	1	horse
S4	CTGAGATACGCG	GTGCCAGCMGCCGCGGTAA	Cruz	0	c	2	horse
S5	ACTACGTGTGGT	GTGCCAGCMGCCGCGGTAA	Cruz	7	c	2	horse
S6	AGTGCGATGCGT	GTGCCAGCMGCCGCGGTAA	Cruz	14	c	2	horse
S7	CCGACTGAGATG	GTGCCAGCMGCCGCGGTAA	Cruz	21	c	2	horse
S8	CCTCTCGTGATC	GTGCCAGCMGCCGCGGTAA	Cruz	28	c	2	horse
S9	CATATCGCAGTT	GTGCCAGCMGCCGCGGTAA	Boston	0	r	3	horse
S10	ACGATGCGACCA	GTGCCAGCMGCCGCGGTAA	Boston	7	r	3	horse
S11	CGTGCATTATCA	GTGCCAGCMGCCGCGGTAA	Boston	14	r	3	horse
S12	AGCTATCCACGA	GTGCCAGCMGCCGCGGTAA	Goose	0	c	4	horse
S13	ATGCAGCTCAGT	GTGCCAGCMGCCGCGGTAA	Goose	7	c	4	horse
S14	CTAACGCAGTCA	GTGCCAGCMGCCGCGGTAA	Goose	14	c	4	horse
S15	CACGTGACATGT	GTGCCAGCMGCCGCGGTAA	Goose	21	c	4	horse
S16	CTCAATGACTCA	GTGCCAGCMGCCGCGGTAA	Goose	28	c	4	horse
S17	ACAGTTGCGCGA	GTGCCAGCMGCCGCGGTAA	Diego	0	r	5	horse
S18	ATCGATCTGTGG	GTGCCAGCMGCCGCGGTAA	Diego	7	r	5	horse
S19	CACGACAGGCTA	GTGCCAGCMGCCGCGGTAA	Diego	14	r	5	horse
S20	CTCGTGGAGTAG	GTGCCAGCMGCCGCGGTAA	Vegas	0	c	6	horse
S21	AGTGTCACGGTG	GTGCCAGCMGCCGCGGTAA	Vegas	7	c	6	horse
S22	CAAGTGAGAGAG	GTGCCAGCMGCCGCGGTAA	Vegas	14	c	6	horse
S23	GCGTTACACACA	GTGCCAGCMGCCGCGGTAA	Vegas	21	c	6	horse
S24	CATCGTATCAAC	GTGCCAGCMGCCGCGGTAA	Vegas	28	c	6	horse
S25	GAACTGTATCTC	GTGCCAGCMGCCGCGGTAA	Ribs	0	r	7	horse
S26	CAGTGTCAGGAC	GTGCCAGCMGCCGCGGTAA	Ribs	7	r	7	horse
S27	CTGGACTCATAG	GTGCCAGCMGCCGCGGTAA	Ribs	14	r	7	horse
S28	ATCTTAGACTGC	GTGCCAGCMGCCGCGGTAA	Uno	0	c	8	horse
S29	GAGGCTCATCAT	GTGCCAGCMGCCGCGGTAA	Uno	7	c	8	horse
S30	CAGACATTGCGT	GTGCCAGCMGCCGCGGTAA	Uno	14	c	8	horse
S31	CGATGCACCAGA	GTGCCAGCMGCCGCGGTAA	Uno	21	c	8	horse
S32	GATACGTCCTGA	GTGCCAGCMGCCGCGGTAA	Uno	28	c	8	horse

Fig. 31 Mapping (txt) file containing metadata for both experiments.

```
Data Bodyweight;
Title 1 'MB2016_01 Effect of restricting time allowed for grazing on body weight';
Input horse $ trt $ t1 t2 t3;
        BW = t1; time = 1; output;
        BW = t2; time = 2; output;
        BW = t3; time = 3; output;

        drop t1-t3;
datalines;
Vegas  C    630   655   662
Uno    C    596   594   603
Cruz   C    493   508   517
Goose  C    532   549   558
Apollo R    657   649   626
Diego  R    586   585   585
Ribs   R    555   549   519
Boston R    632   628   624

;
proc print;

run;
```

Fig. 32 SAS code for determining differences in BW in EXPT 1.

```
Data FecalpH;
Title 1 'MB2016_01 Effect of restricting time allowed for grazing on fecal pH';
Input horse $ trt $ baseline t1 t2 t3;
        pH = t1; time = 1; output;
        pH = t2; time = 2; output;
        pH = t3; time = 3; output;
        drop t1-t3;

datalines;
Goose   c   7.20   7.20   7.41   7.23
Ribs    r   7.21   7.21   7.37   6.99
Uno     c   7.65   7.65   7.35   7.51
Boston  r   7.45   7.45   7.29   7.27
Diego   r   7.05   7.05   .      6.87
Vegas   c   7.38   7.38   7.15   7.37
Apollo  r   6.72   6.72   7.09   7.07
Cruz    c   7.63   7.63   7.40   7.60

;
proc print;

run;

proc mixed data=FecalpH covtest;
        class horse time trt;
        model pH = baseline trt time trt*time;
                repeated / subject = horse (trt) type = CS r rcorr;
                lsmeans trt time trt*time /pdiff ;
run;

quit;
```

Fig. 33 SAS code for determining differences in fecal pH in EXPT 1. Diego d 7 is a missing value due to an insufficient amount of feces.

```
Data FecalpH;
Title 1 'MB2016_01 Effect of diet change over time on fecal pH';
Input horse $ t1 t2 t3 t4 t5;
        pH = t1; time = 1; output;
        pH = t2; time = 2; output;
        pH = t3; time = 3; output;
        pH = t4; time = 4; output;
        pH = t5; time = 5; output;
        drop t1-t5;

datalines;
Goose  c       7.20    7.41    7.23    7.28    7.31
Uno    c       7.65    7.35    7.51    7.50    7.46
Vegas  c       7.38    7.15    7.37    7.30    7.27
Cruz   c       7.63    7.40    7.60    7.54    7.51

;

proc mixed data=FecalpH covtest;
        class horse time;
        model pH = time;
                repeated / subject = horse (time) type = un r rcorr;
                lsmeans time /pdiff ;
run;
```

Fig. 34 SAS code for determining differences in fecal pH in EXPT 2.

```
Data total VFA;
Title 1 'MB2016_01 Effect of restricting time allowed for grazing on totvfa';
Input horse $ trt $ baseline t1 t2 t3;
        tot = t1; time = 1; output;
        tot = t2; time = 2; output;
        tot = t3; time = 3; output;

        drop t1-t3;
datalines;
Cruz    c    61.97    61.97    56.00    56.14
Goose   c    52.13    52.13    56.31    53.17
Uno     c    61.57    61.57    58.10    53.77
Vegas   c    67.98    67.98    61.49    51.33
Apollo  r    60.95    60.95    56.73    56.12
Boston  r    49.93    49.93    48.51    49.38
Diego   r    49.17    49.17     .       48.35
Ribs    r    55.41    55.41    45.09    46.97

;
proc print;

run;

proc mixed data=total covtest;
        class horse time trt;
        model tot = baseline trt time trt*time;
                repeated / subject = horse (trt) type = ar(1) r rcorr;
                lsmeans trt time trt*time /pdiff ;
run;

quit;
```

Fig. 35 SAS code for determining differences in fecal VFA profiles in EXPT 1. Diego d 7 is a missing value due to an insufficient amount of feces.

```
Data acetate;
Title 1 'MB2016_01 Effect of diet change over time on acetate';
Input horse $ t1 t2 t3 t4 t5;
        ac = t1; time = 1; output;
        ac = t2; time = 2; output;
        ac = t3; time = 3; output;
        ac = t4; time = 4; output;
        ac = t5; time = 5; output;

datalines;
Cruz    60.4492    57.2875    58.0843    56.7448    55.0591
Goose   52.8761    56.1339    54.8137    55.2603    54.6564
Uno     57.7003    54.9242    54.3552    53.8413    56.3362
Vegas   62.3901    60.1154    53.9083    51.2160    51.2160
;

proc mixed data=acetate covtest;
        class horse time;
        model ac = time;
                repeated / subject = horse (time) type = un r rcorr;
                lsmeans time /pdiff ;
run;
```

Fig. 36 SAS code for determining differences in fecal VFA profiles in EXPT 2.

```
Data Firmicutes;
Title 1 'MB2016_01 Effect of restricting time allowed for grazing on Firmicutes RA';
Input horse $ trt $ t1 t2 t3;
        F = t1; time = 1; output;
        F = t2; time = 2; output;
        F = t3; time = 3; output;

        drop t1-t3;
datalines;
Apollo r        33.8    27.4    25.4
Boston r        38.3    30.4    30.2
Diego  r        34.9    29.7    29.8
Ribs   r        34.6    25.8    18.1
Cruz   c        39.4    32.3    27.9
Goose  c        30.8    28.4    28.9
Uno    c        50.6    21.9    32.0
Vegas  c        42.9    30.0    33.9

;
proc print;
run;

proc mixed data=Firmicutes covtest;
        class horse time trt;
        model F = trt time trt*time;
                repeated / subject = horse (trt) type = ar(1) r rcorr;
                lsmeans trt time trt*time /pdiff ;
run;

quit;
```

Fig. 37 SAS code for determining differences bacterial phyla in EXPT 1.

```
Data Bacteriodetes;
Title 1 'MB2016_01 Effect of diet change over time on Bacteriodetes';
Input horse $ t1 t2 t3 t4 t5;
        B = t1; time = 1; output;
        B = t2; time = 2; output;
        B = t3; time = 3; output;
        B = t4; time = 4; output;
        B = t5; time = 5; output;
        drop t1-t5;
datalines;
Cruz    37.30   53.60   47.10   41.60   46.40
Goose   46.00   60.00   52.50   48.20   45.60
Uno     33.90   61.10   53.10   49.60   44.50
Vegas   40.40   54.00   49.40   44.20   44.40

;

proc mixed data=Bacteriodetes covtest;
        class horse time;
        model B = time;
                repeated / subject = horse (time) type = AR(1) r rcorr;
                lsmeans time /pdiff ;
run;
```

Fig. 38 SAS code for determining differences bacterial phyla in EXPT 2.