ABSTRACT

PEARSON, AMANDA CATHERINE. Effect of Non-Structural Carbohydrate Intake from Forage on Hindgut Fermentation Parameters in Horses. (Under the direction of Dr. Paul Siciliano, Dr. Vivek Fellner, and Dr. Gerald Huntington).

Two experiments were conducted to determine the effect of nonstructural carbohydrate (NSC) intake on indicators of hindgut pH and fecal fermentation characteristics. Experiment 1 (EXPT1) was conducted to evaluate the effects of grazing fall pasture (relatively high NSC concentration) versus hay (low NSC concentration). Experiment 2 (EXPT2) was conducted to determine the effect of grazing spring pasture in the morning (low NSC concentration) versus afternoon (higher NSC concentration).

In EXPT1 six geldings were randomly assigned to one of two dietary treatments in Period 1 (P1): pasture fed (PF; n = 3) or hay fed (HF; n = 3). PF horses had access to non-toxic endophyte infected tall fescue pasture from 1300 to 0700 the following d, followed by stall confinement from 0700 to 1300 each d for 14 d. HF horses were individually offered non-toxic endophyte infected tall fescue hay (2% of body weight, as-fed) one time per d at 1300. This continued for 14 d. Treatments were then switched for an additional 14-d period, Period 2 (P2), so that all horses received both treatments. Fresh fecal samples were collected from each horse on d-14 of each period to determine fecal pH and to inoculate fecal batch cultures. Pasture grass samples reflecting what the horses ate were taken on d4-7 and d11-14 of each period and analyzed for chemical composition using NIR. Differences in response variables (fecal pH, VFA profiles, body weight) between treatments within horses were analyzed using a paired t-test. Mean pasture NSC concentrations were 11.26 ± 1.4%. Mean

hay NSC concentrations were 6.80% NSC. Overall mean fecal pH (±SEM) was 7.49 ± 0.07 for HF and 7.20 ± 0.06 for PF and was statistically different between treatments (P = .04). Mean batch culture media total VFA concentration ± SEM was 85.25 ± 3.39mM for PF and 73.43 ± 5.76 mM for HF, and were not statistically different between treatments (P = .18).

In EXPT2 six geldings were randomly assigned to one of two treatments: morning turnout (AM; n = 3) or afternoon turnout (PM; n = 3). The AM and PM groups were turned out every d at 0700 and 1230h respectively, and allowed to graze for 8h. Following the first 14-d period, treatments were switched for an additional 14-d period, so that all horses received both treatments. The AM and PM horses grazed non-toxic endophyte infected tall fescue in separate, adjacent grazing cells. Horses were housed in individual stalls while not grazing. Fresh fecal samples were collected from each horse on d-12 to determine fecal pH inoculate batch cultures. Pasture grass samples reflecting what the horses ate were taken on d9-11 of each period and analyzed for chemical composition using NIR. Response variables (fecal pH and VFA) were analyzed using analysis of variance for cross-over designs and pasture NSC concentration was analyzed using analysis of variance for repeated measures design. Mean NSC increased (P < .01) from 15.2 ± .7% at 800hr to 21 ± .7% at 1400hr and did not change further at 2000hr during P1, and was not different between sampling times during P2, resulting in a significant period and treatment x period interaction. Mean batch culture media total VFA concentration was higher (P = .005) for AM (81.6 ± 1.4 mM) compared to PM (70.4 ± 1.4 mM); and higher (P = .004) in P1 (82 ± 1.4 mM) compared to P2 (70 ± 1.4 mM). Mean fecal pH was not affected by period (P = .5) or treatment (P = .72).

Effect of Non-Structural Carbohydrate Intake from Forage on Hindgut Fermentation Parameters in Horses

by
Amanda Catherine Pearson

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APPROVED BY:

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Dr. Vivek Fellner Dr. Gerald Huntington

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Dr. Paul Siciliano

Chair of Advisory Committee

DEDICATION

This thesis is dedicated to my family, soon to be family, and friends, who have encouraged me through this entire process. Without their love and support I would not be where I am today. I would especially like to thank my Mom and Dad, who taught me from an early age to work hard, have fun, and shoot for the moon. I would also like to say a special thanks to my sister and my fiancé who were always there to put a smile on my face when that was what I needed most over the past few years. Without the continuous support and encouragement of each and every one of you I would not be where I am today. I am truly blessed to have such a great support system, and the words "Thank You" are not even close to enough to express my gratitude.

BIOGRAPHY

Amanda Catherine Pearson grew up in Roanoke Rapids, North Carolina, daughter of Gordon and Tracy Pearson. She always had a love for animals, especially horses, and knew she wanted to go to school to learn more about them from a very early age. She never had a horse of her own, but rode them every chance she was given. She graduated from Roanoke Rapids High School in 2005, and that summer moved to Raleigh, North Carolina to attend North Carolina State University. Amanda majored in Animal Science and minored in Nutrition. Her junior year in undergraduate school she decided that she wanted to stay on at North Carolina State University to obtain a Master's Degree. So after graduating in the spring of 2009 with a Bachelor of Science, she continued her education at North Carolina State University's Graduate School to obtain her Master's of Science in Animal Science and minor in Nutrition under the guidance of Dr. Paul Siciliano.

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CHAPTER I. LITERATURE REVIEW

Plant Carbohydrate Fractions

Carbohydrates in plants can be categorized as structural carbohydrates (SC) or nonstructural carbohydrates (NSC). Structural carbohydrates include the carbohydrates in the cell wall, such as cellulose, hemicelluloses, and pectins. Nonstructural carbohydrates include the cell content carbohydrates, such as simple sugars, starch and fructan [1]. Fructans are the major storage carbohydrate of cool season grasses (C3) [1], and are made in the leaf of the plant and are then translocated to the stem for storage. Fructans are polymers of fructose with a terminal sucrose. The way the fructose molecules are linked to one another defines the type of fructan. Those with mostly or exclusively β(2-1) linkages are inulins [2]. They have a degree of polymerization between 2 and 60 [2] and are found in dicotyledonous plants [3]. Those fructans with mostly or exclusively β(2-6) linkages with a degree of polymerization <100 are phleins [2], and are mainly found in small grains [4]. Oligofructans are in the inulin family, with a degree of polymerization of 2 to 20 [2]. This lower degree of polymerization makes them more rapidly fermentable by microbes in the hindgut [5]. Inulins and fructooligosaccharides are the primary fructan types used experimentally to induce laminitis via nasogastic bolus. However, horses mostly consume phleins in pasture grasses [3].

Starch is the major storage carbohydrate of warm season, C4 grasses and legumes [6,7]. Starch storage differs from fructan storage in that starch is produced and stored in the

chloroplast, and this process is self limiting (unlike the storage of fructan), which keeps high amounts of starch from accumulating in warm season grasses and legumes. The lack of self limitation in the storage of fructan allows for the possibility of high amounts of fructans to accumulate in the stems of cool season grasses. Therefore, NSC content of cool season grasses tends to be higher than NSC content of warm season grasses [1].

NSC content is also affected by the plant's environment. Reduced light, warmer temperatures, and moist, fertile soil decrease NSC content (due to a reduction in photosynthesis and accelerated plant growth), while increased sunshine, drought and cool temperatures increase NSC content, especially fructan (due to an increase in photosynthesis coupled with a decrease in plant growth, which leads to more carbohydrates being stored in the steam and chloroplasts of the plant) [8]. Waite et al, 1953 [8] conducted a two-year experiment (1951-1952), in which both years differed in terms of rainfall and temperature, and they noted differences in NSC concentration between the two years. They found that in the spring of 1952 warmer temperatures and less rainfall promoted growth more than the higher rainfall and much cooler temperatures did in 1951. They found grass grew taller and faster in the spring of 1952 vs. 1951, going from 10.2cm in April, 1952 to 61cm in June 1952, vs. 1951 when fescue grew from 10.2cm to ~23cm from April to June [8]. This caused for a greater NSC concentration in May/June of 1951 (~10% to ~22% DM), than in 1952 (~2% to ~5%). This shows that NSC concentrations increase during times of little or no growth, and then concentrations drop during times of increased growth. Waite et al in 1953 [8] found fructan fluctuation within each year in accordance with plant growth. They found

in 1951 that fructosan dropped in the stems of fescue from Mid-May (~22% of DM) to Mid-June (~10% of DM). These were times when they found the plants to go from a vegetative state to floral development, so fructan storage stopped and stores were used during this high-energy demand period for the plant [8]. Hoffman et al [9] also found evidence that NSC% varies greatly during the year. They found that from September of 1995 to November of 1996 that rapidly fermentable NSC fluctuated throughout the year with large increases from September (~9.2%NSC) to October (~10.8%NSC), the peak of NSC% being in November (~11.0%NSC), the nadir in February (~3.8%NSC), and then another large increase from February to March (~7.9%NSC) [9]. In a study done in Blackstone, VA, pasture samples (containing mostly orchard grass) were analyzed once a week from April 7 to June 2, 2007 [10]. They found that water soluble carbohydrate (WSC-the sum of simple sugars and fructan [1]), and fructan individually, were highest in late April and then decreased in May and June [10]. These abrupt changes in NSC concentrations throughout the year could cause harm to the horse since abrupt changes do not allow them to acclimate to higher NSC concentrations slowly over time.

Besides seasonal changes, NSC content can very within a day, generally rising through the morning and maxing out in the afternoon as photosynthesis occurs, and then a decline to lows overnight when respiration occurs [10]. In the study done in Blackstone, VA by Kagan et al [10], pasture samples were taken between 0800 and 1000 in the morning, and between 1600 and 1700 in the afternoon. WSC content was higher in the afternoon versus the morning on 5 out of 8 collection dates. The overall average NSC content was 13.6 ±

0.3% DM in the morning and 15.8 ± 0.3% DM in the afternoon. The average fructan concentration was 7.1 ± 0.3% DM in the morning and 8.3 ± 0.3% DM in the afternoon. The lowest NSC concentrations (May5, May 26, and June 2) were preceded by overall increases in temperature (daily high temperatures and daily low temperatures), which supports that warmer temperatures decrease NSC content, due to presumed increases in plant growth causing the need for the use of storage carbohydrates as a carbon source. In summary, non-structural carbohydrate concentrations in grasses are influenced by the environment (i.e. rainfall and sunshine) which can affect photosynthesis: plant growth ratio, coupled with the varying energy demands of the plant during different stages of growth throughout the year.

Process of Digestion in Horses

In the fundic region of the stomach and in the small intestine of horses there is normally 10^8-10^9 bacteria/g of contents [11]. Bacteria that life in the stomach are those that can life in moderate acidity, such as *lactobacilli*, *streptococci*, and *Veillonella gazogenes* [11]. Proteolytic bacteria are 30-fold or more greater in the small intestine than in the cecum and large intestine [12], and about 19.7% of the bacteria in the cecum are thought to be proteolytic [13]. The flora of the cecum and colon numbers about $0.5x10^9$ to $5x10^9$/g of content. Protozoa number about 55.5 ± 20.5 x 10^3/mL of cecal contents and about 93.24 ± 42.5 x 10^3/mL of colon contents [14]. Additionally studies have shown that the protozoa are mainly from the following genera: *Buetschlia*, *Cyloposthium*, *Blepharocorys*, and *Paraosptricha* [14]. Protozoa contribute less to digestion, and therefore metabolism, than

bacteria, even though they are larger. Anaerobic Gram-positive bacteria are the main class of bacteria in the cecum (80%) [13].

The population percentage of microorganisms may change by 100-fold during a 24h period. This is due to the availability of nutrients (i.e. starch and protein), that once metabolized change the pH of the medium [11]. In the cecum, a pH of 6.5 has been suggested to be the optimum pH for microbial activity and VFA absorption [11]. The VFA produced during fermentation in the cecum would quickly make the cecum an unsuitable environment for microbial growth, due to the decrease in pH that is caused by VFA production, therefore a healthy environment is maintained by the absorption of these VFA into the bloodstream. In the cecum, when the pH moves closer to the pK of a VFA, absorption of that VFA is increased. The H+ ions required for this are believed to be derived from mucosal cells in exchange for NA+. Bicarbonate is a buffer that is secreted into the lumen in exchange for Cl-. Thus, absorption of VFA is accompanied by a net absorption of NaCl [11].

In comparison, microbial fermentation of carbohydrates in the hindgut of cattle is responsible for 5 to 10% of total tract carbohydrate digestion and volatile fatty acid production also varies greatly depending on dietary, animal, and/or environmental factors [15]. When these factors contribute to abnormal or excessive flow of fermentable carbohydrates from the small intestine, hindgut acidosis can occur [15]. Gressley defines hindgut acidosis as an accumulation of organic acids and a subsequent decrese in digesta pH and dramatic shifts in microbial populations that may cause damage to the animal and it is

often indicative of failure of healthy ruminal function [15]. It is characterized by increased rates of production of short-chain fatty acids (including lactic acid), decreased digesta pH, and damage to gut epithelium [15], very similar to the process that is believed to happen in the horse. Over 95% of the VFA produced in the hindgut are passively absorbed across the intestinal epithelium [16], accounting for 8-17% of total VFA absorbed from the digestive tract of ruminants [17]. VFA absorption rates increase with decreasing pH [18]. While bicarbonate passes across the epithelium into the rumen and hindgut to assist in buffering, the hindgut is a fermentation compartment with less buffering capacity than the rumen due to the lack of saliva in the hindgut [19], a lack of protozoa which sequester rapidly fermentable carbohydrates and slow the pH decline after a meal [20,21], making the hindgut less capable of maintaining digesta pH during times of increased VFA production [15]. Also the mucus layer covering the intestinal mucosa results in a microclimate with a pH near 7 that is fairly unresponsive to changes in digesta pH, therefore increased VFA absorption maybe not happen when digesta pH lowers and VFA concentrations increases [20]. Additionally since some VFA absorption is bicarbonate dependent, failure of decreased digesta pH to increase VFA absorption would decrease bicarbonate transfer to the hindgut, further reducing buffering capacity [15]. This reduced digesta pH can then cause a shift in microbial populations, both of which can cause damage to gut epithelium, however the precise pH decline that results in this has not yet been fully identified [15]. These events in the hindgut may contribute to laminitis and other health conditions [15], similar to what is believed to happen in the horse.

The rumen and large colon of cattle and the cecum and large colon of the horse have often been compared due to their similarities in function; however, they are not entirely similar. The cecal microorganisms in the horse tend to be about 1/3 less efficient at digesting organic matter and crude fiber than the ruminal microbes in cattle [22]. This is believed to be due to different cellulolytic microbial species in the cecum of the horse versus those in the rumen of cattle, and due to a more rapid rate of passage of digesta through the horse GI system [11]. Kern et al [13] found that the number of cellulolytic bacteria were greater in the rumen (70.3×10^6) than the cecum (43.0×10^6). Protozoa were also found to be in greater numbers in the rumen of the steer at 10.2×10^4/ml versus that in the cecum of ponies (5.7×10^3/ml), and the protozoa present in the rumen were of different types than those present in the cecum. The steer rumen had approximately threefold more viable bacteria than the pony cecum, with the steer rumen having 16.6×10^8/g ingesta versus the pony cecum having 4.9×10^8/g ingesta [13]. Kern et al found that the cecum had more gram-positive and gram-negative rods than the rumen (70.2% vs. 35.5%) and also more E. Coli (2.6/g ingesta x 10^{-4} vs. <0.5/g of ingesta x 10^{-4}), although percentage of E. coli was low in the rumen and the cecum due to the inhibiting qualities of VFAs. Kern et al also found that the total VFA concentrations were greater in the cecum (97.4µM/g of ingesta) than in the rumen (58.6µM/g of ingesta).

NSC Digestion

In the equine digestive system, there are multiple areas of carbohydrate digestion. The equine stomach contains some microbes that are capable of fermenting carbohydrates and it is possible that some fructan can be hydrolyzed to some extent here as well by gastric acids [1]. Kern et al [13] found 20-fold more viable anaerobic bacteria in the fundic region of the stomach than in the pyloric region, this coupled with the presence of VFAs seen by Kern et al and lactate seen by Healy et al [23] supports the belief that some fermentation happens in the stomach of the equine [13].

Many of the carbohydrates (like starch) are hydrolyzed and absorbed as monosaccharides in the small intestine. When there is an excess of hydrolyzable carbohydrate, that is too much for the small intestine to digest, the excess starch is then passed on to the cecum [1], to be fermented along with the carbohydrates that can't be broken down by mammalian enzymes (like fructan). Potter et al 1992[24] suggested that the capacity for the small intestine for starch digestion is a starch intake of 3.5 to 4 g/kg BW. Fermentation of carbohydrates by microbes in the cecum yields volatile fatty acids (VFAs) as a byproduct, with acetate being the main VFA produced and propionate and butyrate also having significant production [25] [26]. These VFAs are metabolically important in that they account for 30% of a horse's daily digestible energy (DE) intake [27]. However when there is starch overload in the hindgut (due to excess starch intake in the diet), this can lead to rapidly fermented carbohydrates, leading to an excess of lactate and propionate production, which can increase the risk for certain diseases in the horse, such as colic and laminitis [9].

Fructans cannot be broken down by mammalian digestive enzymes because of their β 2-1 linkages. Since β2-1 linkages cannot be digested by mammalian digestive enzymes, they are fermented by microbes in the cecum [2]. Berg et al [28] shows data that supports the idea that fructooligosaccharides are digested in the hindgut. They saw that fructooligosaccharide supplementation (8-24g/d) led to a linear increase in fecal total VFA production and lactic acid concentration, giving proof that the bacteria in the hindgut are indeed digesting these carbohydrates, since there are more byproducts present [28]. When starch intake exceeds 0.4% of body weight per feeding, the excess starch can also be digested in the hindgut [24].

The carbohydrate composition of a plant is also important because it can affect the microbe population in the horse's hindgut, as well as affect the VFA's these microbes produce. For example, bifidobacteria (often associated with "good bacteria") can use fructooligosaccharides as an energy source, leading to VFA's and lactic acid as by products, lowering the pH, which prevents the growth of pathogenic bacteria [28]. Studies have also shown that fructooligosaccharide ingestion has led to an increase in bifidobacteria in humans[29] and pigs [30]. Berg et al [28] did a study with 9 quarter horse yearlings that were on one of three treatments, no fructooligosaccharides (FOS) (CON treatment), 8g FOS/d (LOW treatment), and 24gFOS/d (HIGH treatment). Treatments were supplemented once a day mixed with grain before the morning feeding of concentrate supplement, after which horses had free access to pasture, fresh water, and a plain salt block. Horses were also giving a concentrate supplement at night. The trial had 3 consecutive 10d feeding periods, with one horse from each group receiving a different treatment during each 10-d period, such

that all horses received all treatments. On the last 3 d of each 10-d period, fecal samples were collected from the surface of a rubber-matted stall floor during the 0730 feeding. Microbial analysis of *lactobacilli, Escherichia coli, and Bifidobacteria* populations were run. They concluded that the optimal dose of fructooligosaccharide supplementation daily is between the range of 8-24g to decrease pathogenic bacteria in the intestines of yearlings, due to a decrease in *E.coli* in the LOW treatment and there being no difference in *E. coli* between the CON and HIGH treatments [28]. This, coupled with studies done in humans [31], rats [32] and other species that shows a significant positive correlation between fructooligosaccharide ingestion and *Bifidobacteria*, suggests that fructooligosaccharide ingestion my promote healthy bacteria in the gut, which in turn decreases harmful bacteria in the gut.

However, there is a distinctive line between the amount of carbohydrate ingestion that can benefit the health of the horse, and the amount that can lead to less desirable results. When a horse ingests a moderate-very high percentage of carbohydrates, especially NSC's, carbohydrate fermentation can happen very quickly, which can outweigh the benefits of carbohydrate in the diet and cause problems for the horse, since their gut health is essential to their overall well being [28]. Garner et al [33] gave 17.6g of cornstarch flour gruel (85% corn starch, 15 wood cellulose flour) per kg body weight to 31 horses to see if laminitis would be induced. 23 out of 31 horses showed signs of laminitis, two of which died [33]. High rates of fermentation can lead to an overgrowth of lactobacilli and streptococci (gram positive bacteria) which can cause changes in the hindgut which could interfere with the

growth of other bacteria in the hindgut as well as lead to an increase in lactic acid production [34]. These are factors that are believed to trigger laminitis in the horse [34].

Additionally, carbohydrate overload can be a problem in horses that are predominately stabled, since often they are receiving two larger meals per day, versus eating small meals frequently throughout the day (as they would if they were maintained on pasture) [35]. Garner et al found that oral administration of starch induced symptoms similar to laminitis in horses [33]; therefore these "bolus" meals could have the same effect. Bolus meals high in carbohydrate content could provide a horse with more carbohydrate than its small intestine is able to digest at one given time, thus dumping the excess carbohydrate into the hindgut, along with the carbohydrate that is already being fermented there. This carbohydrate overload can lead to the laminitis trigger factors previously mentioned (accelerated VFA production, especially lactic acid, leading to acidosis and laminitis) [34]. Bolus meals may also increase the rate of passage of feed through the horse's digestive system, which may lead to decreased digestion and absorption of other nutrients [35] that cannot be fermented as quickly as the carbohydrates. Also since increased feed equals a proportionally greater passage rate, this would support that bolus meals could lead to more carbohydrates being fermented in the cecum, passed on passage rate alone. And if these meals also contain a large percentage of carbohydrates the effects could be even more pronounced.

NSC and Laminitis

Laminitis is a painful condition that is common among equines and can lead to permanent injury or death [36]. In the United States laminitis is the second most common reason for a horse to be presented to a veterinarian (with the most common reason being colic) [37]. In the UK they experience about 8000 cases of laminitis per year, and about 600 of these have to be euthanized due to the severity of the laminitis, with another 1300 horses left permanently unsound [37]. There are many predisposing trigger factors that can cause a horse to become laminitic, most of them with nutritional basis. Additionally, not all equines are equally affected by laminitis, suggesting that some horses may be predisposed to the condition [38]. Horses that have had laminitis are believed to be predisposed to have it again.

Laminitis occurs in three stages: the developmental phase, the acute phase, and the chronic phase. In the developmental phase lamellar separation is triggered [39], and there are no visible symptoms of pain. This is a very important phase because it is the only phase in which the effects can be prevented and possibly reversed. However, this phase only lasts for 8 to 40 hours after exposure to the laminitis triggers. In a healthy horse, the lamellar architecture of the hoof is responsible for suspending the distal phalanx inside the hoof. In a laminitic horse, the distal phalanx begins to separate from the inner hoof wall after detachment is triggered, which causes this infrastructure to fail and the weight of the horse will force the bone down into the hoof capsule. This begins the process of damaging arteries

and veins, crushing the corium of the sole and coronet, thereby causing the debilitating pain and other symptoms experienced by these horses [39].

The acute stage is characterized as beginning at the onset of the clinical lameness until the point at which there is evidence of displacement of the distal phalanx within the hoof capsule [39]. Once in this phase horses can have a complete recovery, die (or have to be euthanized) from laminitis complications, or develop chronic laminitis. The chronic phase of laminitis is noted by the presence of palmar/plantar displacement of the distal phalanx. This phase can continue on indefinitely, and the horses in this phase may or may not regain the ability to perform or work at the same level or magnitudes they could before they were affected by laminitis [39].

In the lamellar tissue of laminitic horses there is an increase in concentration of enzymes (metalloproteinase-2, also known as MMP-2 and metalloproteinase-9, also known as MMP-9) [40] that are normally found in healthy lamellar tissues [41]. In healthy horses, MMP enzymes help to maintain hoof structural integrity in response to daily stressors. In laminitic horses the concentrations of MMP enzymes in the lamellar tissue can be up to double that of normal lamellar tissue [39], however the exact mechanism for increases in MMP concentration in laminitic horses is yet to be explained [42]. Some researchers suggest that vasoconstriction can impede blood flow in the hooves, leading to ischemic necrosis of epidermal lamellae [43], while other studies found lamellar vessels to be normally dilated in carbohydrate-induced laminitis [42].

It is believed that laminitis can be induced by the overconsumption of fermentable carbohydrates found in lush pasture or cereal grains [36] [44]. A survey conducted in the UK found that the majority of horse owners believe that most cases of laminitis occur in animals on pasture [45], most likely due to the ingestion of excess NSC. Fructans are believed to be the form of carbohydrate in pasture that are involved in the onset of acute laminitis [46]. It is believed that excessive fructan fermentation results in the production of laminitis trigger factors (such as lactic acid, and amine compounds), which then enter the horse's circulation, inevitably leading to laminitis. Endotoxins [47] and MMP [48] have also been found to be trigger factors of laminitis in horses. Mungall et al [42] has investigated the proposed causes of equine laminitis in vitro, by investigating the force required to separate epidermal from dermal lamellae once the tissues were incubated with laminitis trigger factors. Through this model, they have found that the lamellae have been resistant to almost all known cytokines, tissue factors and prostaglandins. However, when the tissues were subjected to *Streptococcus bovis* cultures, MMP-2 within the hoof wall was activated and caused lamellar separation [42]. Crawford et al [36]fed fructan as inulin at a rate of 3g/kg of BW/day (broken up into 3 meals to mimic pasture feeding) to a group of 12 ponies (1/2 of which were predisposed to laminitis). None of these ponies showed signs of laminitis, suggesting that fructan ingestion leading to laminitis is above 3g/kg of BW/day. However they did see a dose-dependent change in fecal pH after 24h of inulin digestion (pH=6.89 on hay diet to minimum of pH=6.18 on inulin diet) and formation of vasoconstrictive amines (2-2.5 fold increase for 6 of 14 amines identified and quantified in the fecal samples). However, this

fecal pH drop (0.75 of a log unit) was small compared to a mean 3 log units decrease reported in horses where laminitis was induced by carbohydrate overload from a bolus feeding of oligofructose at a rate of 7.5-12.5g/kg of BW [49]. Additionally, in the study by Crawford et al [36], plasma lactate and amines were not altered by fructan ingestion of 3g/kg of BW/day, leading to the belief that the hindgut mucosa stayed intact and laminitis trigger factors were not allowed to enter into circulation, which is why they didn't see laminitis symptoms. This is most likely due to the fact that the hindgut pH did not drop low enough to negatively affect the intestinal mucosa, allowing it to stay intake, keeping laminitis trigger factors such as lactic acid and vasoconstrictive amines inside of the intestines. Crawford et al [36] also did not see any significant differences between the 2 groups of horses in fecal pH and amine production, suggesting that differences among individual horses that predispose some to laminitis may involve other factors than intestinal microflora or mucosal barrier, including barrier function of the intestinal mucosa, detoxifying capacity of enzymes, diabetes, etc [36]. In the study by van Eps and Pollitt 2006 [49] they fed fructans in bolus feedings via nasogastric tube to 18 horses at a much higher rate, 7.5-12.5g/kg of BW of oligofructose depending on treatment. All horses fed oligofructose developed laminitis. Along with a significant decrease in fecal pH they also saw an increase in heart rate, rectal temperature, digital pulse, and plasma D-lactate. Plasma D-lactate is only produced by bacteria, so it's presence in the blood is a marker of hindgut fermentation taking place, as well as the ability of laminitis trigger factors to enter the blood stream through weakened intestinal walls due to lowered pH. Plasma D-lactate increase coincided with a decrease in

fecal pH. Additionally the onset of clinical laminitis occurred after the peak in plasma D-lactate. Fecal pH stayed just above 7.1 in the control group, but dropped rapidly (pH=4.2-4.6) in all treatment groups 5-12 hours post bolus feeding and remained low until 20-24 hours post bolus feeding, after which fecal pH started to slowing increase back towards a pH of 7. Based on their study they suggest an administration dose of 10g/kg BW/day of oligofructose in future experiments to induce laminitis. French and Pollitt, 2004 [50] found that a range of oral fructan administration of 7.5–12.5 g/kg BW induced laminitis in horses. These dosages would coincide with an intake which a horse on pasture could theoretically consume. Longland et al 1999 [51] found that fructan intake from the perennial ryegrass pasture could range from 2.5–10.5 g fructan/kg BW, assuming a DM intake of 2.5 % of BW for a 500-kg horse. However, smaller dosages of fructan also may alter the hindgut environment and increase risk of laminitis. In ponies, the consumption of inulin at 1 g or 3 g/kg BW resulted in a significant decrease in fecal pH [36]. Vervuert et al. [52] reported fructan concentrations in cool-season grass-legume pasture ranging from 18 ± 8 to 57 ± 17 g/kg DM. Assuming a DM intake of 2.5% of BW, the fructan intake from the cool-season grass-legume mix pasture studied by Vervuert et al. [52] is considerably lower (0.45–1.4 g fructan/kg BW) than the range reported capable of inducing laminitis by French and Pollitt, 2004 [50] (7.5-12.5g/kg BW). Hoffman et al. [9] reported that the rapidly fermentable carbohydrate content of grass-legume pasture (a large proportion of which was assumed to be fructan) ranged from 22.9–145 g/kg DM. This could result in an intake of approximately 0.57–3.6 g rapidly fermentable carbohydrate/kg BW (assuming DM intake of 2.5 % of BW),

which is also considerably lower than the laminitis inducing range suggested by French and Pollitt [50]. Additionally ranges found from experiments which induce (or try to induce) laminitis through a bolus feeding of oligofructose or other fructans are not easily compared to the actual ranges that horses maintained on pasture could consume. This is due to the fact that horses on pasture take in small amounts of feed throughout the day, causing them to consume smaller doses of fructan throughout the day, versus large, bolus amounts all at once. Smaller intakes of fructan throughout the day could lead to less rapidly fermented carbohydrates entering the hindgut at one time, which may keep carbohydrate overload from happening even when fructan concentrations are high. The grazing habits of horses that would already potentially lower the amount of fructan being consumed at one time could have an even bigger impact if horses were allowed to graze during times of the day and year when fructan and other NSC concentrations are low. These types of grazing strategies could help decrease the risk of laminitis, colic, etc. for horses that are out on pasture.

CHAPTER II: EXPERIMENT 1

2.1 Introduction

A single large nasogastic bolus of non-structural carbohydrate (NSC; provided as fructooligosaccharide) consistently induces laminitis in horses [49]. The mechanism is thought to involve accelerated microbial fermentation, accumulation of volatile fatty acids (VFA) and lactic acid, a subsequent decrease in hindgut pH causing mucosal damage and allowing entry of microbial derived laminitis "trigger factors" [49].

Non-structural carbohydrates (NSC) are a highly fermentable component of cool season pasture grasses that fluctuate throughout the year and peak in spring and fall [5]. Additionally, NSC rise through the day, peaking in late afternoon and fall over night to lows in early morning [5].

Limited information exists regarding the effect of NSC intake from cool season grass pasture on microbial fermentation parameters and hindgut pH. A greater understanding of these effects will contribute to laminitis prevention strategies for grazing horses. Therefore an experiment was designed to test the hypothesis that microbial fermentation will be accelerated, VFA profiles will be altered and hindgut pH will be decreased in horses grazing fall pasture having relatively high NSC concentrations as compared to horses fed similar forage in hay form having lower NSC concentration. Our objectives were to compare fecal pH, total VFA concentration and VFA profiles (within a batch culture using fecal inoculums) between horses grazing fall pasture containing relatively high NSC concentration or fed hay having lower LSC concentration.

2.2. Materials and Methods

The following experimental protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC approval # 08-084-A).

Animals and Experimental Design

Six stock-type geldings ranging in age from 5 to 8 yr and weighing 553 ± 37 kg (mean ± SD) were used in a completely randomized two-period switch-back design to determine the effect of grazing fall pasture having moderate non-structural carbohydrate concentration (NSC; 11.3 ± 1.4%), or fed hay (made from the same pasture) having lower NSC concentration (6.8%), on parameters reflecting hindgut pH and fermentation characteristics. Horses in this experiment were maintained on grass pasture 6 mo prior to the start of the experiment.

Horses were randomly assigned to one of two dietary treatments: pasture fed (PF; n = 3) or hay fed (HF; n = 3). All horses were individually housed in 3.7 x 12.2m stalls. Each pen was partially covered (~50%). Horses were acclimated to pens prior to the start of the experiment for 7 days. The PF group had daily access to non-toxic endophyte infected tall fescue pasture (*Festuca arundinacea*, Max-Q, Pennington Seed, Madison, GA) from 1300 to 0700 the following day, followed by stall confinement from 0700 to 1300, for a period of 14d. The HF horses were offered tall fescue hay (harvested one year prior to the study from the same pasture that horses in the PF group grazed) individually at a rate of 2% of body weight (as-fed) one time per d at 1300. Remaining hay was removed from stalls at 0700 the following morning. HF horses were then re-fed 2% of body weight at 1300. This continued for 14 d. Treatments were then switched for an additional 14-d period so that each horse received both treatments. Two separate, but adjacent pastures, each measuring approximately 144 x 56 m, were used in the first and second periods of the experiment.

Therefore each period began with a pasture that had not been previously grazed. All horses were weighed on d 1 and 14 of each period at approximately 1230 using an electronic scale (Smart Scale 200, Gallagher Animal Management, USA). All horses had unlimited access to water in both stalls and pasture. The only supplemental feed horses received was 225g of alkane treated oats twice daily at 0700 and 1900 associated with a parallel study using alkanes as markers of digestibility and fecal output in order to estimate pasture intake [53].

Sample Collection and Analysis

DMI (dry matter intake) of PF in this study was found in a parallel study using alkanes as markers of digestibility and fecal output in order to estimate pasture intake [53]. DMI of HF horses was determined by direct measurement of how much hay HF ate per d over the entire 14-d period.

Herbage mass of each pasture was estimated one day prior to starting each of the two experimental periods, and again on the final day of each experimental period. Herbage mass was estimated using a plate drop meter [54] [55]. Briefly, 85 to 100 measures were recorded from each pasture using the falling plate meter for herbage mass estimation. Readings were taken every 4-6 steps while walking in a serpentine pattern over each pasture. The falling plate meter was calibrated by identifying and measuring 3 high, 3 medium, and 3 low sward heights within the pasture. A .25 x .25 m square was placed under the plate meter with-in each of the 9 areas (i.e., 3 high, 3 medium, and 3 low sward heights) and the area inside of the square was clipped to 2.5 cm height. To derive the calibration equation DM weight of

forage collected from the 9 samples used in calibration were regressed against the plate height. Each pasture sample was placed in a separate sealed plastic bag for transport to the lab, where samples were weighed, dried in an oven at 60°C to a constant weight (i.e., dried to a constant weight at 60°C). The %DM was calculated as the dry weight/initial weight x 100. Initial herbage mass (IHM) available was then calculated by multiplying herbage mass from the beginning of each period by pasture size in ha. IHM available for grazing was calculated assuming an efficiency of 0.5 (IHM available x 0.5 = IHM available for grazing).

Fresh fecal samples were collected from each horse on d 14 of each period via rectal grab-sampling, or from the ground, with care to prevent contamination, if defecation occurred prior to rectal collection, at 0700 on d14 of each period. Fecal samples were only collected from the ground from horses prior to rectal collection when defecation was seen (while collections were being done on other horses), to ensure collections were fresh. A portion of the feces were used to determine fecal pH, and the remainder was used to inoculate batch cultures.

Fecal pH was measured according to Crawford 2007 [36]. Each horse's fecal sample used for pH measurement was placed individually in sealed plastic bags (n=6) upon collection and then immediately placed into a water bath (37°C). Within 1 h after collection 50 g of feces from a single horse was placed in a 150 ml plastic container and filled with 50 ml of 37°C DI water (1 container per individual horse's fecal sample). Lids were placed back on the containers and the samples plus deionized (DI) water were mixed for 1 minute using a vortex (VWR Signature Digital Vortex Mixer, Radnor, PA). Three pH readings were

made on each sample (Symphony pH portable meter VWR Instruments, Radnor, PA), and an average pH was calculated. The pH meter was calibrated using a two-point calibration (pH 4.01 and 7) to ensure accuracy prior to making measurements.

The remaining fecal samples from each horse were immediately placed in a thermos after collection to maintain a temperature near 37ºC and were then transported to the lab within 1 hour of collection. These fecal samples were used to inoculate batch cultures [56], that were used as indicators of overall microbial activity and to detect any shifts in microbial populations.

The batch culture method was performed by adding 50g of feces to a 125 ml Wheaton bottle with a screw cap containing 1g of alfalfa pellets and an artificial saliva buffer solution [57]. The alfalfa pellets acted as the microbial substrate. The bottle was then capped (topped off) with CO2, sealed and placed in a water bath set at 39°C for 48 h. After 48 h of incubation in the water bath, bottles were removed from the water bath and placed in an ice water bath in order to stop fermentation. Batch culture bottles were analyzed for methane production by taking a 10 µl gas sample from the bottles using a gas tight syringe (Hamilton Co., Reno, Nevada). This sample was then run ran through a GC model CP-3800 Varian (Walnut Creek, CA) with a molsieve %A 45/60 mesh stainless steel column (Supelco, Bellefonte, PA). Batch cultures bottles were analyzed for VFA by GLC (gas liquid chromatography) equipped with a FID (flame ionization detector) on the Varian CP-3380 (Walnut Creek, CA) using a fused silica capillary column, 30m X .25mm X .25um film thickness (Nukol; Supelco Inc., Bellefonte, PA). Per sample this was done by pipetting 1ml

of fluid from the bottle and placing it in a tube to which 200 µl of 25% meta-phosphoric acid with internal standard (2-ethyl butyric acid) was then added. (Standards were calibrated using an internal standard.) The sample was then centrifuged at 21,000 rcf (IEC Micromax bench top centrifuge) for 10 minutes after which it was ready for GLC analysis. Lactate concentrations were also measured on the same samples processed for VFA analysis using a commercial assay kit (K-607-100, Biovision, Mountainview, CA) based on a spectrophotometric technique.

Hay samples were taken using a core sampler and were taken for each period (n=2). Samples were taken from multiple bales of hay (n=6+) to ensure the final sample was a good estimation of hay used in the study. Hay samples were then sent to the NCDA for analysis.

Pasture grass samples reflecting grazing height of horses were collected on d 4-7 and d 11-14 of each period and analyzed for chemical composition (including NSC) using NIR [58]. Samples were collected at 1600 h on d 4, at 0630 and 1600 h on d 5 and 6, at 0630 h on d 7, at 1600 h on d 11, at 0630 and 1600 h on d 12 and 13, and lastly at 0630 h on d 14. Samples were collected by monitoring horses for approximately 5 minutes to determine the grazing height and forage type being consumed. All samples for each time period dried using paper towels to get rid of all external moisture and were then placed in individual gallon sized, sealed, plastic bags, and immediately placed in an Igloo cooler for the walk from the pasture to the lab, and then stored at 20ºC. Once all samples for the period were taken, samples were placed back into a cooler, and then transported by car to the NSCU laboratory. Grass samples were freeze-dried over 96 h. Grass samples were then compiled

according to period, day of collection, and collection time so that grass samples were now in 8 bags (P1/d4-d6PM, P1/d5-d7AM, P1/d11-d13PM, P1/d12-d14AM, P2/d4-d6PM, P2/d5-d7AM, P2/d11-d13PM, P2/d12-d14AM). Then a subsample was taken from each bag and ground through a 5mm screen on a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA), and then another subsample was taken and ground through a 0.5mm screen in a Cyclotec 1093 mill (Rose Scientific Ltd, Edmonton, Alberta, Canada). NIR [58] was then done on the ground samples to determine NSC, CP, NDF, and ADF.

Statistical Analysis

Differences in response variables (fecal pH, VFA profiles, body weight) between treatments within horses were analyzed using a paired t-test (H.J. Motulsky, Prism 5 Statistics Guide, 2007, GraphPad Software Inc., San Diego CA, www.graphpad.com). Results are expressed as the mean of differences between treatments within horses (± SEM). A value of $P < 0.05$ was considered significant.

2.3. Results

Chemical Composition of Pasture and Hay

Mean pasture chemical composition (for all sampling times, n=8) was 22.57 ± .61 % DM, 15.6 ± .29% CP, 56.09 ± 1.14% NDF, 30.85 ± .45% ADF, and 11.26 ± 1.4% NSC.

Pasture DE, as calculated from pasture chemical composition according to Pagan, 1998 [59] was 2.27 ± .03% Mcal DE/kg DM. Mean pasture carbohydrate fractions for P1 and P2 were as follows: NSC: 11.36 ± 1.36% and 12.58 ± 1.36% for P1 and P2 respectively and were not significantly different (P = .44). Pasture glucose was 3.278 ± .2% and 3.294 ± .2% for P1 and P2 respectively, and were not significantly different (P = .95). Pasture starch was 1.39 ± .23% and .99 ± .23% for P1 and P2 respectively and were not significantly different (P = .16). Pasture monosaccharide was 5.77 ± .67% and 6.38 ± .67% for P1 and P2 respectively and were not significantly different (P = .44). Mean hay chemical composition (for all sampling times, n=2) was 93.85 ± .15% DM, 11.92 ± 1.59% CP, 56.09 ± 1.80% NDF, 35.55 ± .05% ADF, and 6.80% NSC. Hay starch, calculated by difference of water soluble carbohydrate (WSC, 6.3%) from NSC (6.8%), was .5%. Hay DE, as calculated from hay chemical composition according to Pagan, 1998 was 2.00 ± .01% Mcal DE/kg DM.

Mean BW between treatments

Mean BW of horses receiving the hay and pasture treatments was 549 ± 41kg and 559 ± 39 kg, respectively. When horses were on the PF treatment they were 9.3 (95% confidence interval: -15.4 to -3.3) kg heavier (P = .011) than when on the HF treatment.

DM Intake

Mean DM intake of hay was 9.09 ± 1.02 kg/d.

Initial Herbage Mass and Availability

Initial herbage mass (IHM) was 2810 ± 87 kgDM/ha for P1 and 2112 ± 61 kgDM/ha for P2. IHM was significantly different between periods ($P < .0001$). Initial herbage mass available for grazing was 1133kgDM (2.03kgDM/kgBW) for P1 and 851.6kgDM (1.52kgDM/kgBW) for P2.

Fecal pH, VFA, Lactate, and Methane Production.

All results (minus lactate and methane production) are shown in Table 1. The pasture treatment resulted in decreased fecal pH ($P = .04$) and propionate molar % ($P < .01$); whereas it resulted in an increased ($P < .01$) butyrate and valerate molar percent. Treatment did not affect total VFA concentration and the molar percent of acetate, isobutyrate, and isovalerate. Methane production was 11.65 ± 1 nmol/ml and 12.55 ± 1 nmol/ml for the pasture and hay treatments respectively and was not significantly different ($P = .41$). Lactate concentrations were .0036 ± .00045 nmol/ml and .0042 ± .00045 nmol/ml for pasture and hay treatments respectively and were not significantly different ($P = .23$), however these values were below the limits of detection for the assay used (lower limit .02mM).

Table 1: Fecal pH, total VFA, and individual VFA production

Item	Treatment[a]		Mean Difference	Upper 95% CI	Lower 95% CI	Paired t-test p-value
	Hay	Pasture				
Fecal pH	7.49 ± .07	7.2 ± .06	.3	.02	.58	.04
Total VFA mM	73.43 ± 5.8	85.25 ± 3.39	-11.82	-31.45	7.82	.1825
Acetatic Acid[b]	65.18 ± 1.6	64.98 ± .19	.2	-3.681	4.078	.901
Propionic Acid[b]	27.18 ± 1.5	22.08 ± 1.4	5.1	2.8	7.42	<.01
Butyric Acid[b]	6.08 ± .96	9.01 ± .89	-2.93	-4.31	-1.55	<.01
Isobutyric Acid[b]	.3 ± .11	.35 ± .11	.06	-.43	.54	.7815
Valeric Acid[b]	.24 ± .16	3.28 ± .63	-3.04	-4.4	-1.68	<.01
Isovaleric Acid[b]	.27 ± .05	.14 ± .07	.13	-.15	.41	.28

[a] Mean ±SEM, [b] Molar %

2.4. Discussion

Horses are hindgut fermenters with little to no post fermentative digestion and absorption of microbial cells (unlike ruminants), so horse feces should be a readily available source of viable microorganisms, making their feces a good source of microorganisms for in vitro studies [60]. In ruminant studies, in vitro study techniques have used microbial

inoculums prepared from rumen digesta from fistulated animals [61]. However, it has also been shown in ruminants that gut microorganisms closely associated with plant debris in the rumen are also excreted with plant residues in the feces [62,63]. Additionally, fecal material has been shown to remain largely anaerobic after voiding and the microflora can be viable for several hours post void [64]. Additionally, for horses, using feces as the microbial inoculums for in vitro studies would be more advantageous over cecal digesta because it is a cheap, readily available source of microorganisms that does not require fistulated animals [60]. It also minimizes the effects of variation between animals due to the fact that feces can be collected from individual or several animals [60]. Feces from sheep and cattle have also been successful replacements for rumen digesta as inoculums for in vitro digestibility studies [65,66]. This information coupled with the fact that horses are hindgut fermenters with little to no post fermentative digestion and absorption, as previously stated, gives reason why using horse feces as microbial inoculums for in vitro studies is a logical choice to asses cecal fermentation. This information is supported by the methane production in the batch culture fermentation bottles used in this study which proves that there was indeed fermentation taking place within the bottles that used horse feces as microbial inoculums.

While IHM was significantly different between treatments in this study, IHM available for grazing was not limiting in either treatment, therefore horses were able to consume as much pasture as they wanted regardless of treatment. Mean DM intake of pasture was found as a part of parallel study and was 14.36 ± 2.62 kg/d [53]. Therefore NSC intake was .62 kg/d for hay and 1.61 kg/d for pasture. The effect of differing NSC intakes

from the two treatments had a variable affect on hindgut fermentation parameters. Although the increased NSC intake of PF versus HF horses was not associated with a significant increase in hindgut fermentation as indicated by the lack of difference in batch culture total VFA concentration in PF versus HF horses; the total VFA concentrations did numerically increase in 4 out of 6 horses used in this study. This coupled with the slightly reduced fecal pH also seen in this study suggests that consumption of pasture with a higher NSC concentration did have some influence on fermentation parameters in the hindgut. This agrees with other studies that also saw a decrease in fecal pH with increased NSC intake [28,36].

In this study, slightly reduced fecal pH among PF and HF coincides with Berg et al, 2005 [28] and Crawford et al, 2007 [36] who also saw decreases in fecal pH in response to dietary NSC supplementation. Additionally, Kern et al [67] found that fecal pH reflects hindgut pH, suggesting that our differences in fecal pH between treatments, could indicate changes in hindgut fermentation. In this study fecal pH decreased an average of 0.3 log units for PF horses as compared to HF, but fecal pH was still neutral or slightly above. Considerably lower fecal pH (i.e. 4.5) is associated with laminitis induced by alimentary carbohydrate overload [49]. If pasture NSC concentrations had been greater than the moderate concentrations we observed, it is possible that hindgut fermentation would have been more greatly altered and a lower fecal pH may have been observed. Additionally our results may have been influenced by relatively low horse numbers as the total VFA concentration did increase numerically in 4/6 horses fed pasture versus hay. Therefore, an

experiment designed with a larger number of horses may have greater power and therefore may have more statistically significant results regarding total VFA concentration production from batch culture media that may fully support the hypothesis in this experiment.

While acetate concentrations were not significantly different between treatments, there was a decrease in acetate concentrations in PF horses. The lack of change in acetate concentrations between treatments coupled with the increase in butyrate concentrations might suggest metabolism was shifting away from propionate. Butyrate serves as a hydrogen-sink. Therefore it seems more butyrate would be produced in a low pH environment [68]. In studies butyrate has been shown to be absorbed at a greater rate than propionate and acetate (butyrate>propionate>acetate) [69]. Aschenbach et al [69] found an increase in ruminal pH (from a pH of 5.8 that is associated with SARA) associated with an increase in uptake of butyrate (and acetate). So, when pH is low the rumen would prefer to convert pyruvate into acetate/butyrate (versus the opposing pathway of lactate/propionate), since butyrate is readily absorbed and will help get ruminal pH back to optimal levels [68]. This information agrees with data found in this study that found increased proportions of butyrate (with decreased proportions of propionate) with a decrease in fecal pH, suggesting that the hindgut was attempting to adjust its pH by converting more of the carbohydrates to butyrate versus pH lowering lactate/propionate. This information could also explain why acetate concentrations were not increasing, i.e. acetate may have increased but was ultimately converted to butyrate, therefore no net increase in acetate was observed. Also, the in vitro fermentation going on within the bottles did not allow for absorption, so when acetate accumulated in the bottles,

butyrate synthesis was increased, but could not be absorbed, leading to the increase in butyrate concentration that we observed. This idea is also supported by the variable acetate concentrations between horses in the HF treatment versus that in the PF treatment. This could mean that variability was decreased in PF horses because hindgut pH was decreasing so microbes were able to concentrate fermentation on acetate/butyrate production to restore/increase hindgut pH, while in HF horses hindgut pH was already increased so production of VFA's was spread out amongst acetate, butyrate, propionate, etc. Studies have also shown that butyrate is the preferred energy source of colonic epithelial cells [70] and causes greater stimulation of epithelial cell growth in the jejum and distal colon than did propionate and acetate [71], suggesting that butyrate has an important role in the maintenance of intestinal health. This may mean that increased butyrate concentrations that were associated with pasture intake in this trial may actually be a benefit.

NSC values for grass pasture reported in this study fall near the middle of previously reported ranges (i.e. 6 to 20% DM) [72] and can therefore be considered moderate. It has been suggested (with unclear evidence) that an NSC content of less than ~11% is a safe zone for NSC intake [46]. The NSC concentrations in this study for hay (6.8%) were well within this safe zone, and pasture NSC concentrations were just slightly above (11.26%). Additionally, starch concentrations were higher in PF versus HF treatments by approximately .5-.9%. Future experiments should attempt to target pasture grasses with higher NSC concentrations, to help determine where the limit for NSC intake safe zones should actually be. Also the PF DM intake of 14.36 ± 2.26kg/d [53] coupled with differences in NSC

concentration allowed PF horses to consume approximately 1kg/d more NSC than HF horses, 1.62kg/d versus 0.62kg/d respectively. So if grass pasture was grazed with a higher NSC content, then the difference between PF NSC intake and HF NSC intake would also be greater, potentially leading to a greater difference in hindgut fermentation characteristics, including increased lactate production in PF treatments, which could lead to adverse effects in PF horses. Additionally, horses in this study consumed approximately 0.1g/kgBW and 0.36g/kgBW of starch in HF and PF treatments, neither of which is close to the 3.5-4g/kgBW that is believed to be the minimum amount needed to induce starch overload in the hindgut [24].

In conclusion, our results show that hindgut pH and microbial fermentation parameters do not appear to be negatively affected when horses graze fall pasture containing moderate NSC concentrations. However, the results of this study should be considered with care as there were a small number of horses. While total VFA concentration did not significantly increase in PF versus HF horses, it did increase in 4 of the 6 horses. This may mean that fermentation was increased with a greater NSC intake, but that this experiment may not have enough horses to show a significant increase and/or NSC concentrations high enough in our PF treatment to show significant increase in Total VFA concentration. Perhaps experiments with more horses and higher NSC concentrations in pasture and/or greater differences in NSC concentrations between treatments would expand on this experiment, since our results did show an increase in fermentation in 4 of 6 horses with increased NSC intake.

CHAPTER III: EXPERIMENT 2

3.1. Introduction

Non-structural carbohydrates (NSC) are a highly fermentable component of cool season pasture grasses that fluctuate seasonally, peaking in spring and fall [5]. Additionally, NSC rise through the day, peaking in late afternoon and fall over night to lows in early morning [5].

Excessive NSC intake has been implicated in pasture laminitis. One proposed mechanism by which NSC contribute to laminitis is through accelerated hindgut fermentation leading to accumulation of volatile fatty acids and subsequent reduction in hindgut pH, associated mucosal damage and subsequent entry of microbial derived laminitis-trigger factors [6]. Therefore, grazing strategies that reduce NSC intake and potential alterations in the hindgut microbial environment may be useful in the prevention of pasture laminitis. One possible strategy is to restrict pasture access to times of the day when pasture grass NSC concentrations is low (i.e. morning).

Therefore an experiment was designed to test the hypothesis that microbial fermentation will be accelerated and hindgut pH will be decreased in horses turned-out for 8 h beginning in the afternoon (1230-2030 h) versus morning (0700-1500 h) due to greater NSC intake by horses turned-out beginning in the afternoon. Our specific objectives were to compare fecal pH, total VFA concentration and VFA profiles (within a batch culture using fecal inoculums) between horses turned out in the morning vs. afternoon.

3.2. Materials and Methods

The following experimental protocols were approved by the North Carolina State University Animal Care and Use Committee (Protocol 08-084-A). The experiment began on 15 April 2010.

Animals and Experimental Design

Six stock-type geldings ranging in age from 5 to 8 yr and weighing 553 ± 37 kg (mean ± SD) were used in a completely randomized two-period cross-over design to determine the effect of turn-out time on pasture NSC intake and hindgut fermentation characteristics. Horses were initially randomly assigned to one of two dietary treatments: a.m. turnout (AM; n=3) and p.m. turnout (PM; n=3). The AM and PM groups were turned out every morning at 0700 and 1230 respectively, and were allowed to graze for 8h. A single 1.62ha pasture was divided equally into 4 grazing cells approximately 0.41ha in size. The pasture contained mostly non-toxic endophyte infected tall fescue (*Festuca* arundinacea, Max-Q, Pennington Seed, Madison, GA). The AM and PM horses were grazed in separate, adjacent grazing cells. Horses rotated grazing cells every 7d. Horses were housed in individual pens (3.7m x 12.2m) between grazing times. Each pen was partially covered (~50%). Horses were acclimated to pens prior to the start of the experiment. Following the first 14d period, treatments were switched for an additional 14d period, so that all horses received all treatments. The only supplemental feed horses received was 225g of alkane treated oats twice daily at 0700 and 1900 associated with a parallel study using alkanes as

markers of digestibility and fecal output in order to estimate pasture intake [53]. Horses were maintained on grass pasture 6 mo prior to the start of the experiment. All horses were weighed using an electronic scale (Smart Scale 200, Gallagher Animal Management, USA), approximately 30 min to 1 hr prior to turnout time on d-1 and d-12 of each period.

Sample Collection and Analysis

Herbage mass of each pasture was estimated one day prior to starting each of the two experimental periods, and again on the final day of each experimental period. Herbage mass was estimated using a plate drop meter [54] [55]. Briefly, 85 to 100 measures were recorded from each pasture using the falling plate meter for herbage mass estimation. Readings were taken every 4-6 steps while walking in a serpentine pattern over each pasture. The falling plate meter was calibrated by identifying and measuring 3 high, 3 medium, and 3 low sward heights within the pasture. A .25 x .25 m square was placed under the plate meter with-in each of the 9 areas (i.e., 3 high, 3 medium, and 3 low sward heights) and the area inside of the square was clipped to 2.5cm height. To derive the calibration equation DM weight of forage collected from the 9 samples used in calibration were regressed against the plate height. Each pasture sample was placed in a separate sealed plastic bag for transport to the lab, where samples were weighed, dried in an oven at 60°C to a constant weight (i.e., dried to a constant weight at 60°C). The %DM was calculated as the dry weight/initial weight x 100.

Fresh fecal samples were collected from each horse on d 12 of each period via rectal grab-sampling, or from the ground, with care to prevent contamination, if defecation

occurred prior to rectal collection. Fecal samples were collected approximately 30 min to 1 h prior to pasture turnout in each group. Fecal pH was measured according to Crawford 2007 [36], for each individual fecal sample. Each horse's fecal sample used for pH measurement was placed individually in sealed plastic bags (n=6) upon collection and then immediately placed into a water bath (37°C). Within 1 h after collection 50 g of feces from a single horse was placed in a 150 ml plastic container and filled with 50 ml of 37°C DI water (1 container per individual horse's fecal sample). Lids were placed back on the containers and the samples plus DI water were mixed for 1 minute using a vortex (VWR Signature Digital Vortex Mixer, Radnor, PA). Three pH readings were made on each sample (Symphony pH portable meter VWR Instruments, Radnor, PA), and an average pH was calculated. The pH meter was calibrated using a two-point calibration (pH 4.01 and 7) to ensure accuracy prior to making measurements.

The remaining fecal samples from each horse were immediately placed in a thermos after collection to maintain a temperature near 37ºC and were then transported via car to the NCSU Dairy Nutrition Lab within 1 hour of collection. These fecal samples were used to inoculate batch cultures [56], that were used as indicators of overall microbial activity and to detect any shifts in microbial populations.

The batch culture method was performed by adding 50g of feces to a 125 ml Wheaton bottle with a screw cap containing 1g of alfalfa pellets and an artificial saliva buffer solution [57]. The alfalfa pellets acted as the fermentation substrate. The bottle was then capped (topped off) with CO2, sealed and placed in a water bath set at 39°C for 48 h. After 48 h of

incubation in the water bath, bottles were removed from the water bath and placed in an ice water bath in order to stop fermentation. Batch culture bottles were analyzed for methane production by taking a 10 µl gas sample from the bottles using a gas tight syringe (Hamilton Co., Reno, Nevada). This sample was then run ran through a GC model CP-3800 Varian (Walnut Creek, CA) with a molsieve %A 45/60 mesh stainless steel column (Supelco, Bellefonte, PA). Batch cultures bottles were analyzed for VFA by GLC (gas liquid chromatography) equipped with a FID (flame ionization detector) on the Varian CP-3380 (Walnut Creek, CA) using a fused silica capillary column, 30m X .25mm X .25um film thickness (Nukol; Supelco Inc., Bellefonte, PA). Per sample this was done by pipetting 1ml of fluid from the bottle and placing it in a tube to which 200 µl of 25% meta-phosphoric acid with internal standard (2-ethyl butyric acid) was then added. (Standards were calibrated using an internal standard.) The sample was then centrifuged at 21,000 rcf (IEC Micromax bench top centrifuge) for 10 minutes after which it was ready for GC analysis. Lactate concentrations were also measured on the same samples processed for VFA analysis using a commercial assay kit (K-607-100, Biovision, Mountainview, CA) based on a spectrophotometric technique. Body weight measurements were also taken on d 1 and 14 of each period.

Pasture grass samples reflecting grazing height of horses were taken at 0800, 1400, and 2000 hr on d 9, 10 and 11 of each period and analyzed for chemical composition (including NSC) using NIR [58]. Samples were collected by monitoring horses for approximately 5 minutes to determine the grazing height and forage type being consumed.

All samples for each time period dried using paper towels to get rid of all external moisture and were then placed in individual gallon sized, sealed, plastic bags, and immediately placed in an Igloo cooler for the walk from the pasture to the lab, and then stored at 20ºC. Once all samples for the period were taken, samples were placed back into a cooler, and then transported by car to the NSCU laboratory. Grass samples were freeze-dried over 96 h. Grass samples were then compiled according to period and collection time so that grass samples were now in 6 bags (P1/0800h, P1/1400h, P1/2000h, P2/0800h, P2/1400h, P2/2000h). Then a subsample was taken from each bag and ground through a 5mm screen on a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA), and then another subsample was taken and ground through a 0.5mm screen in a Cyclotec 1093 mill (Rose Scientific Ltd, Edmonton, Alberta, Canada). NIR [58] was then done on the ground samples to determine NSC, CP, NDF, and ADF.

Statistical Analysis

Differences in response variables (fecal pH, VFA profiles, body weight, mean pasture NSC concentrations) between treatments were analyzed using analysis of variance for cross-over design according to Walker [73] . The model included treatment sequence, treatment (turn out time), horse within a treatment, and period. Changes in pasture NSC concentration, over a 24-h period regardless of treatment, were analyzed using analysis of variance for repeated measure design [73]. The model included period, time, and period x time

interaction. Results are expressed as least squares means (± SEM). A value of $P < 0.05$ was considered significant.

3.3. Results

Chemical Composition of Pasture

For all sampling times n=6 unless otherwise stated. Mean pasture chemical composition was 29.2 ± .76% DM, 10.8 ± .61% CP, 56.7 ± .91% NDF and 30.08 ± .71% ADF. Mean DE calculated [74] from pasture chemical composition was 2.3 ± .17% Mcal DE/kgDM. NSC data are shown in Table 2. In P1 overall mean NSC increased ($P < .01$) from 800 to 1400 and did not change between 1400 and 2000. Period 1 NSC concentrations were greater ($P < .01$) than P2. A period x time interaction occurred due to greater ($P < .05$) NSC concentrations at times 1400 and 2000 in P1 as compared to time 1400 and 2000 in P2. Forage glucose concentration data are shown in Table 3. Forage glucose concentrations were not significantly different in P1 than in P2 ($P < .42$). Overall mean forage glucose concentrations for P1 and P2 did not differ over time. Overall mean monosaccharide concentrations were significantly different between P1 (6.07 ± .4%) and P2 (4.31 ± .32%) ($P < .05$). Overall mean monosaccharide concentrations in P1 and P2 did not differ over time. Starch data are shown in Table 5. Overall mean starch concentrations were not significantly different between P1 (3.44 ± .2%) and P2 (3.17 ± .16%) ($P = .37$). Overall mean starch concentrations in P1 and P2 did not differ over time.

Mean BW between treatments

Mean BW for AM and PM treatments was 565 kg and 570.7 kg (± 1.31 kg) respectively (P = .04).

Initial Herbage Mass.

Mean IHM ± SE was 1,036 ± 52.55 kg/ha for AM treatments and 1,110 ± 32.23 kg/ha for PM treatments. Mean IHM was not significantly different between treatments (P = 0.32).

Fecal pH, VFA, Lactate, and Methane Production.

Fecal pH and VFA data are shown in Table 6 (minus Lactate and Methane). Mean fecal pH was not significantly affected by treatment (P = .72) or period (P = .5). Mean fecal pH was 7.1 ± .17 and 6.99 ± .17 for AM and PM treatments respectively, and 7.12 ± .17 and 6.94 ± .17 for P1 and P2 respectively. Mean batch culture total VFA concentrations were affected by treatment (P < .01) and by period (P < .01), were higher for AM (81.6 ± 1.4 mM) compared to PM (70.4 ± 1.4 mM), and higher in period 1 (82 ± 1.4 mM) compared to period 2 (70 ± 1.4 mM). Mean batch culture acetate, propionate, and butyrate molar concentrations were not affected by treatment or by period (Table 3). Mean batch culture methane production was 251.8 ± 113.5 nmol/ml and 37.44 ± 113.5 nmol/ml for AM and PM treatments respectively and was not significantly different (P = .12). Lactate concentrations were below the lower limit of detection (lower limit .02mM); and were not significantly

different between treatments (P = .2); .0045 ± .0028 nmol/ml and .0086 ± .0028 nmol/ml for AM and PM treatments respectively.

Table 2: Effect of sampling time on pasture plant NSC concentration within each period[a]

Period	**Sampling time, h**			**SEM**
	0800	*1400*	*2100*	
1	15.2[b]	21.0[cx]	19.4[cx]	.7
2	11.8	12.6	14.8	.7

[a]Overall effect of time, P < .01. Period, P < .001; period = 1, 18.5 ± .3 vs. period = 2, 13.1 ± .3
[bc]Different supper scripts w/ in rows differ P < .05
[x]Different supper script w/ in columns differ P < .05

Table 3: Effect of sampling time on pasture plant glucose concentration within each period

Period	**Sampling time, h**			**SEM**
	0800	*1400*	*2100*	
1	2.4	2.7	2.3	.3
2	2.1	1.9	2.7	.2

Table 4: Effect of sampling time on pasture plant monosaccharide concentration within each period[a]

Period	Sampling time, h			SEM
	0800	*1400*	*2100*	
1	5.6	6.5[b]	6.1	.5
2	3.8[c]	4.3	4.8	.4

[a]Overall effect of period, P < .05; period = 1, 6.07 ± .4 vs. period = 2, 4.31 ± .32
[bc]Different supper scripts differ P < .05

Table 5: Effect of sampling time on pasture plant starch concentration within each period

Period	Sampling time, h			SEM
	0800	*1400*	*2100*	
1	3.5	3.3	3.6	.3
2	3	3.7	2.8	.3

Table 6: Effect of morning (AM) vs. afternoon (PM) grazing on hindgut fermentation characteristics

Item	**Treatment**			**p-value**	
	AM	*PM*	*SEM*	Treatment	Period
Fecal pH	7.08	6.99	0.17	0.72	0.5
Total VFA mM	81.58	70.43	1.42	.005	.004
Acetate molar %	61.02	57.07	1	.11	.46
Propionate molar %	19.12	21.04	2.49	.61	.86
Butyrate molar %	13.84	13.46	1.33	0.9	.31

3.4. Discussion

As previously stated in EXPT1 Discussion, using horse feces as microbial inoculums is practical and advantageous [60]. Methane production was seen in this experiment, as was the case in EXPT1, which again shows us that fermentation did take place in our fecal batch cultures.

The results of this experiment did not directly support our hypothesis. However, the results of this experiment are difficult to interpret due to a lack of change in pasture NSC concentrations between treatments in P2. In P2 the two treatments were basically the same since NSC values were not statistically different between collections taken at 0800h and 2000h, at 0800h and 1400h, and at 1400h and 2000h. Since P1 did have a significant change in NSC concentrations between treatments, P1 results only could potentially be better

interpreted with respects to our hypothesis, but not with great confidence since when only looking at one period n=3 versus 6. However, even when looking at only the P1 results, fecal pH, Total VFA, and molar VFA concentrations were not significantly different (results not shown).

Additionally, in this experiment there was a treatment effect, due to the fact that NSC concentrations were lower in P2 versus P1. This means that horses switching from AM to PM treatments, that should have had an increase in NSC concentrations that they were consuming, actually had a decrease in available NSC. This means that in P1 two distinct treatments were maintained, with low NSC values in the AM and higher NSC values in the PM. However, in P2 there was not a significant difference between the two treatments, and the PM group in P2 actually consumed lower NSC than the AM group in P1. So, in a sense, the period effect has now become a treatment effect, since P1 had high NSC and P2 had low NSC concentrations. This could explain the finding of higher total VFA concentrations in the AM group, but cannot be well explained in light of other findings.

DMI for this study was found in parallel study using alkanes as markers of digestibility and fecal output in order to estimate pasture intake [53]. DMI was 7.48 ± 0.93 kg/d for AM treatments and 9.92 ± 0.93 kg/d for PM treatments, and were not significantly different (P=.1). Additionally mean DM intake was higher in P2 (10.52 ± 0.93 kg/d) compared to P1 (6.88 ± .93 kg/d) (P < .03). Therefore DMI intake coupled with average NSC concentrations per turnout period allowed AM horses to eat approximately 1.67gNSC/kgBW and 2.12gNSC/kgBW in P1 and P2 respectively, while PM horses ate

approximately 3.01gNSC/kgBW and 2.71gNSC/kgBW in P1 and P2 respectively. Thus, increased intake in P2 compensated for lower NSC concentrations in P2, allowing our treatments to somewhat be maintained.

Additionally AM and PM horses ate approximately .46gStarch/kgBW and .54gStarch/kgBW respectively. Both of these intakes are well below the intake of 7.5-12.5g/kgBW (oligofructose-via nasogastric bolus) that French and Pollitt, 2004 [50], or the starch administration of ~17g/kgBW via nasogastric bolus [49] used to induce laminitis. Additionally NSC concentrations for EXPT2 were higher than NSC concentrations in EXPT1; however minimal levels of methane and lactate were produced in both studies. This also suggests that much higher NSC (and potentially starch) intakes are needed to induce laminitis or other detrimental effects from excessive fermentation.

Acetate, Butyrate, and Propionate concentrations were not significantly different between treatment or period, suggesting that fermentation was not altered drastically enough in either period or treatment to individually effect these concentrations. Butyrate concentrations in this study were higher than in EXPT1, further supporting the theory that with lower pH the hindgut will convert more pyruvate to butyrate to try and increase pH back to optimal levels as discussed in EXPT1.

Fecal pH in this study is also similar to (but slightly lower than) the mean fecal pH seen in EXPT1, in which lower overall NSC concentrations were observed. Additionally, no matter which period or treatment, fecal pH values in this study were still in the neutral zone. Also, this implies that hindgut pH is not drastically altered in horses grazing pastures with

NSC concentrations between 11.84% and 21%. These concentrations are above the limit of ~11% NSC suggested by Geor, 2009 [46], suggesting that horses can potentially graze higher amounts of NSC over an extended period of time without detrimental effects. However, it is possible that horses with a predisposed phenotype may be predisposed to pasture-associated laminitis [46], so these horses should consume higher NSC concentrations with great care. Future studies looking at hindgut fermentation characteristics of predisposed horses and/or horses that have had an episode of laminitis in the past grazing pasture in the morning versus the afternoon would likely be beneficial to help determine more realistic NSC "safe zones".

NSC concentrations for P1 of this study are similar to other studies dealing with tall fescue in Virginia that found NSC to be >15%-20% of DM in April and May [46]. However, P2 NSC concentrations were lower for all 3 sampling times. Kagan et al [10] in a study done with orchardgrass found that fructan percentages were highest (11.1%) in April and declined in May and June to as low as 1.4%. They also saw a great deal of variability between collections per month with percentages going from 1.4 to 4 to 2.8 from May 5-May 19. Kagan et al [10] also saw lower WSC concentrations in the morning (collection time 8-10) versus the afternoon (1600-1700) , on all collection dates except May 26. This may imply that in this area May is a month that has variable NSC concentrations due to weather that may increase or decrease NSC concentrations. Katz et al [75] also did a study in which the highest prevalence and incidence of laminitis occurred in May. This suggests that the variable and potentially unpredictable NSC concentrations in the month of May may cause horses to be susceptible to getting laminitis from carbohydrate overload.

The results of this study may have been more definitive with more fecal collections for fecal pH and fecal batch culture coupled with more grass collections for NSC concentrations. This would allow for the greatest comparison of the effects of NSC on fecal pH and fecal batch cultures, giving us an idea of what is going on throughout the entire day in the hindgut in response to changes in NSC intake, versus what's going on at two specific points in the day. Additionally more frequent grass samplings may have better reflected actual NSC intake. Also in this experiment turnout times overlapped (with AM turn-out ending at 1500 and PM turn-out beginning at 1230), so adjusting turn-out times so that the two groups of horses were not on pasture at the same time may show more significant results. Additionally, experiments that have significantly different NSC concentrations between treatments, and also have similar AM and PM values between periods would likely produce more significant and easily interpreted results.

In conclusion, the results of this study did not support our hypothesis that microbial fermentation will be accelerated, VFA profiles will be altered and hindgut pH will be decreased in horses turned-out for 8 hr beginning in the afternoon (1230-2030) versus morning (0700-1500). However, our hypothesis was made on the assumption that horses grazing in the PM versus the AM would be consuming higher amounts of NSC, which was not the case, although DMI did make up for some of the low NSC concentrations that were not expected. So the results of this study should be interpreted with care. More studies should be done taking care that NSC values will be different between treatments for each period, treatments values are similar between periods, and also adjusting turn-out time so that

treatments don’t overlap to see if the implications of this research would be repeated in the actual setting that we were hoping to have but did not achieve due to low NSC values in the second period.

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APPENDIX

Table 7: EXPT1 fecal pH raw data

	Hay	Pasture
Apollo	7.55	7.14
Goose	7.23	7.30
Vegas	7.41	7.21
Diego	7.47	7.38
Cal	7.58	6.99
Uno	7.72	7.15

Table 8: EXPT1 acetic acid raw data

	Pasture	Hay
Cal	64.27	64.99
Goose	65.39	70.13
Diego	64.67	62.72
Vegas	65.24	67.24
Uno	65.41	67.02
Apollo	64.91	58.98

Table 9: EXPT1 propionic acid raw data

	Pasture	Hay
Cal	23.54	29.49
Goose	21.91	25.62
Diego	27.58	32.26
Vegas	18.16	21.49
Uno	22.47	26.25
Apollo	18.81	27.98

Table 10: EXPT1 isobutyric acid raw data

	Pasture	Hay
Cal	0.05	0.35
Goose	0.80	0.00
Diego	0.17	0.25
Vegas	0.42	0.06
Uno	0.29	0.75
Apollo	0.39	0.38

Table 11: EXPT1 butyric acid raw data

	Pasture	Hay
Cal	7.40	4.92
Goose	9.15	4.13
Diego	5.43	3.35
Vegas	10.63	9.06
Uno	10.48	6.45
Apollo	10.94	8.55

Table 12: EXPT1 isovaleric acid raw data

	Pasture	Hay
Cal	0.14	0.48
Goose	0.31	0.00
Diego	0.14	0.11
Vegas	0.42	0.10
Uno	0.27	0.15
Apollo	0.34	0.00

Table 13: EXPT1 valeric acid raw data

	Pasture	Hay
Cal	4.57	0.42
Goose	2.47	0.00
Diego	2.06	0.04
Vegas	4.96	0.98
Uno	1.25	0.00
Apollo	4.34	0.00

Table 14: EXPT1 lactic acid raw data

	Pasture	Hay
Cal	0.0041	0.0046
Diego	0.0051	0.0050
Uno	0.0031	0.0026
Goose	0.0045	0.0047
Vegas	0.0030	0.0040
Apollo	0.0015	0.0041

Table 15: EXPT1 total VFA raw data

	Hay	Pasture
Cal	77.23	78.32
Goose	69.00	75.00
Diego	91.37	80.23
Vegas	74.12	93.60
Uno	79.90	91.69
Apollo	48.98	92.65

Table 16: EXPT1 BW raw data

	Pasture	Hay
Diego	580.	574
Uno	556.	548
Cal	508.	494
Goose	520.	506
Vegas	580.	580
Apollo	608.	594

Table 17: EXPT1 BW data for each collection time

Horse ID	Period	Treatment	t1	t2	t3
Diego	1	Pasture	578	590	580
Uno	1	Pasture	541	558	556
Cal	1	Pasture	506	520	508
Goose	2	Pasture	506	526	520
Vegas	2	Pasture	580	584	580
Apollo	2	Pasture	594	621	608
Goose	1	Hay	520	516	506
Vegas	1	Hay	570	578	580
Apollo	1	Hay	602	598	594
Diego	2	Hay	580	576	574
Uno	2	Hay	556	560	548
Cal	2	Hay	508	508	494

Table 18: EXPT1 plant carbohydrate fractions raw data

NSC/TNC

P1		P2	
AM	PM	AM	PM
11.04	12.156	12.992	16.9
9.594	12.662	8.42	12.026
10.317	12.409	10.706	14.463

Gluc

P1		P2	
AM	PM	AM	PM
3.182	3.269	3.301	3.81
3.163	3.499	2.808	3.255

Starch

P1		P2	
AM	PM	AM	PM
1.356	1.923	0.931	1.273
1.288	1.006	0.499	1.236

Mono

P1		P2	
AM	PM	AM	PM
5.345	5.984	6.013	8.392
4.737	7.031	4.872	6.237

Table 19: EXPT1 methane raw data

Day 14 of trial (Period 1 Day 14)

Bottle Number	Description	Rep	Substrate wt (g)	Methane (48hr-nmol/ml)
1	Horse1 CAL	1	1.01	14.38
2	Horse1	2	1.01	8.22
3	Horse1	3	1.00	12.29
4	Horse1	4	1.00	13.64
5	Horse2 GOOSE	1	1.02	19.75
6	Horse2	2	1.01	24.47
7	Horse2	3	1.00	11.14
8	Horse2	4	1.01	9.66
9	Horse3 DIEGO	1	1.02	13.11
10	Horse3	2	1.01	10.8
11	Horse3	3	1.01	14
12	Horse3	4	1.02	9.89
13	Horse4 VEGAS	1	1.01	9.16
14	Horse4	2	1.03	9.73
15	Horse4	3	1.02	12.39
16	Horse4	4	1.00	12.38
17	Horse5 UNO	1	1.00	9.46
18	Horse5	2	1.01	9.26
19	Horse5	3	1.00	10.54
20	Horse5	4	1.02	11.46
21	Horse6 APOLLO	1	1.01	10.66
22	Horse6	2	1.00	11.14
23	Horse6	3	1.01	11.54
24	Horse6	4	1.01	7.18
25	Control, no feces	1	1.00	10.15
26	Control, no feces	2	1.00	.
27	Horse1 BLANK CAL	1	--	13.81
28	Horse2 BLANK GOOSE	1	--	12.21
29	Horse3 BLANK DIEGO	1	--	9.16
30	Horse4 BLANK VEGAS	1	--	.
31	Horse5 BLANK UNO	1	--	7.38
32	Horse6 BLANK APOLLO	1	--	8.32

Day 28 of trial (Period 2 Day 14)

Bottle #	Sample	Rep	Substrate wt(g)	Methane(nmol/ml)
1	Horse1 DIEGO	1	1.01	14.81
2	Horse1	2	1.00	11.9
3	Horse1	3	1.01	12.27
4	Horse1	4	1.02	11.24
5	Horse2 UNO	1	1.01	13.77
6	Horse2	2	1.02	12.02
7	Horse2	3	1.00	24.55
8	Horse2	4	1.01	10.28
9	Horse3 VEGAS	1	1.01	11.61
10	Horse3	2	1.01	10.27
11	Horse3	3	1.02	9.95
12	Horse3	4	1.02	13.09
13	Horse4 APOLLO	1	1.01	10.49
14	Horse4	2	1.01	10.56
15	Horse4	3	1.00	10.71
16	Horse4	4	1.02	10.52
17	Horse5 CAL	1	1.01	8.85
18	Horse5	2	1.02	12.49
19	Horse5	3	1.00	8.84
20	Horse5	4	1.01	10.98
21	Horse6 GOOSE	1	1.01	11.45
22	Horse6	2	1.00	18.14
23	Horse6	3	1.02	14.96
24	Horse6	4	1.01	10.69
25	Control, no feces	1	1.01	11.71
26	Control, no feces	2	1.01	10.23
27	Horse1 BLANK Diego	1	--	14.26
28	Horse2 BLANK Uno	1	--	12.38
29	Horse3 BLANK Vegas	1	--	14.37
30	Horse4 BLANK Apollo	1	--	13.35
31	Horse5 BLANK Cal	1	--	13.47
32	Horse6 BLANK Goose	1	--	13.14

Table 20: Initial herbage mass data (kg/.25m^2) for prism input for P1, EXPT1

Period	HM	Period	HM	Period	HM
1	0.217796	1	0.212732	1	0.217796
1	0.293756	1	0.205136	1	0.319076
1	0.281096	1	0.255776	1	0.217796
1	0.243116	1	0.212732	1	0.331736
1	0.243116	1	0.306416	1	0.116516
1	0.154496	1	0.255776	1	0.319076
1	0.306416	1	0.460868	1	0.243116
1	0.319076	1	0.382376	1	0.167156
1	0.230456	1	0.369716	1	0.129176
1	0.357056	1	0.293756	1	0.179816
1	0.344396	1	0.293756	1	0.382376
1	0.293756	1	0.382376	1	0.243116
1	0.243116	1	0.395036	1	0.268436
1	0.243116	1	0.357056	1	0.319076
1	0.369716	1	0.331736	1	0.141836
1	0.382376	1	0.167156	1	0.319076
1	0.281096	1	0.293756	1	0.331736
1	0.382376	1	0.268436	1	0.293756
1	0.407696	1	0.319076	1	0.243116
1	0.357056	1	0.217796	1	0.268436
1	0.293756	1	0.116516	1	0.382376
1	0.167156	1	0.192476	1	0.243116
1	0.395036	1	0.167156	1	0.205136
1	0.306416	1	0.319076	1	0.255776
1	0.230456	1	0.369716	1	0.382376
1	0.268436	1	0.369716	1	0.331736
1	0.217796				

Table 21: Initial herbage mass data (kg/.25m^2) for prism input for P2, EXPT1

Period	HM	Period	HM	Period	HM
2	0.222603	2	0.199263	2	0.327633
2	0.280953	2	0.140913	2	0.105903
2	0.187593	2	0.129243	2	0.152583
2	0.187593	2	0.140913	2	0.245943
2	0.199263	2	0.304293	2	0.222603
2	0.140913	2	0.210933	2	0.164253
2	0.187593	2	0.187593	2	0.315963
2	0.257613	2	0.175923	2	0.280953
2	0.187593	2	0.234273	2	0.304293
2	0.280953	2	0.187593	2	0.257613
2	0.409323	2	0.175923	2	0.164253
2	0.199263	2	0.175923	2	0.152583
2	0.327633	2	0.175923	2	0.199263
2	0.245943	2	0.199263	2	0.105903
2	0.374313	2	0.187593	2	0.187593
2	0.187593	2	0.164253	2	0.199263
2	0.187593	2	0.187593	2	0.175923
2	0.187593	2	0.175923	2	0.234273
2	0.187593	2	0.175923	2	0.187593
2	0.129243	2	0.245943	2	0.210933
2	0.245943	2	0.199263	2	0.199263
2	0.187593	2	0.199263	2	0.222603
2	0.199263	2	0.257613	2	0.187593
2	0.187593	2	0.315963	2	0.210933
2	0.187593	2	0.152583	2	0.234273
2	0.222603	2	0.245943	2	0.210933
2	0.199263	2	0.210933	2	0.245943
2	0.210933	2	0.257613		

Table 22: Grass heights (in) for P1 and P2, EXPT1

4.5	4	5.25	2.75	4
6	4.25	9.3	6.75	4.25
5.75	4	7.75	4	3
5	4	7.5	3.5	4
5	4.75	6	4	5.5
3.25	4.25	6	3.75	4
6.25	4.5	7.75	3.75	6
6.5	5.5	8	4.25	8.75
4.75	4.5	7.25	5.5	3.75
7.25	5.25	3.5	6.75	5
7	4.25	6	3.25	4
6	3	5.5	5.25	4.5
5	2.75	6.5	4.5	4.25
5	3	4.5	4.75	4
7.5	6.5	7	4	4.25
7.75	4.5	4	4.5	5.25
5.75	8.25	3.5	5	4.25
7.75	4	7.75	2.25	5.25
7.25	3.75	5	3.25	7.75
6	5	5.5	5.25	6.75
3.5	4	6.5	4.75	6.5
8	3.75	3	3.5	7.5
6.25	3.75	6.5	6.75	7.5
4.75	3.75	4.5	6	6.75
5.5	4.25	6.5	6.5	6
4.5	4.25	4.5	5.5	5
4.25	7	6.75	3.5	5.5
5.25	5.25	2.5	3.25	7.75
4.4	8	6.5	4.25	5
4.25	4	5	2.25	4
5.25	4	3.5	4.75	3.75
4.4	4	2.75	6	4
6.25				

Table 23: Hay intake EXPT1

Horse ID= Goose (Sequence 1)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/7/2009	13.00 (2.5% BW)	13.06	5.95	9.43
10/8/2009	10.4 (2% BW)	10.23	2.64	7.59
10/9/2009	10.4	10.4	3.46	6.94
10/10/2009	10.4	10.32	1.94	8.38
10/11/2009	10.4	10.43	2.02	8.41
10/12/2009	10.4	10.4	1.67	8.73
10/13/2009	10.4	10.4	0.25	10.15
10/14/2009	10.4	10.4	1.3	9.1
10/15/2009	10.4	10.4	0.69	9.71
10/16/2009	10.4	10.4	0.29	10.11
10/17/2009	10.4	10.39	0.42	9.97
10/18/2009	10.4	10.43	0.91	9.52
10/19/2009	10.4	10.4	0.4	10
10/20/2009	10.4	10.4	0.53	9.87

Horse ID= Cal (Sequence 2)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/21/2009		10.32	3.35	6.97
10/22/2009	10.16 (2% BW)	10.32	2.23	8.09
10/23/2009	10.16	10.16	3.52	6.64
10/24/2009	10.16	10.16	3.62	6.54
10/25/2009	10.16	10.15	2.63	7.52
10/26/2009	10.16	10.16	1.2	8.96
10/27/2009	10.16	10.16	1.78	8.38
10/28/2009	10.16	10.16	0.33	9.83
10/29/2009	10.16	10.16	1.97	8.19
10/30/2009	10.16	10.16	1.55	8.61
10/31/2009	10.16	10.16	2.47	7.69
11/1/2009	10.16	10.16	1.98	8.18
11/2/2009	10.16	10.16	1.02	9.14
11/3/2009	10.16	10.16	1.58	8.58

Horse ID= Apollo (Sequence 1)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/7/2009	15.05 (2.5% BW)	15.38	6.56	8.49
10/8/2009	12.04 (2% BW)	12.12	3.75	8.37
10/9/2009	12.04	12.04	3.23	8.81
10/10/2009	12.04	11.98	1.19	10.79
10/11/2009	12.04	12.04	1.19	10.85
10/12/2009	12.04	12.04	1.42	10.62
10/13/2009	12.04	12.04	1.32	10.72
10/14/2009	12.04	12.04	0.7	11.34
10/15/2009	12.04	12.04	0.87	11.17
10/16/2009	12.04	12.04	0.84	11.2
10/17/2009	12.04	12.05	1.29	10.76
10/18/2009	12.04	12.04	1.14	10.9
10/19/2009	12.04	12.04	0.12	11.92
10/20/2009	12.04	12.04	0.55	11.49

Horse ID= Diego (Sequence 2)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/21/2009		11.8	3.89	7.91
10/22/2009	11.60 (2% BW)	11.8	3.53	8.27
10/23/2009	11.6	11.6	3.05	8.55
10/24/2009	11.6	11.62	2.58	9.04
10/25/2009	11.6	11.59	1.58	10.01
10/26/2009	11.6	11.6	7.3	4.3
10/27/2009	11.6	11.6	0.82	10.78
10/28/2009	11.6	11.6	0.64	10.96
10/29/2009	11.6	11.6	1.76	9.84
10/30/2009	11.6	11.6	1.19	10.41
10/31/2009	11.6	11.6	0.57	11.03
11/1/2009	11.6	11.6	1.16	10.44
11/2/2009	11.6	11.6	1.71	9.89
11/3/2009	11.6	11.6	1.01	10.59

Horse ID= Vegas (Sequence 1)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/7/2009	14.25 (2.5% BW)	13.44	2.17	11.27
10/8/2009	11.4 (2% BW)	11.06	0.75	10.31
10/9/2009	11.4	11.4	0.69	10.71
10/10/2009	11.4	11.35	0.49	10.86
10/11/2009	11.4	11.32	0.37	10.95
10/12/2009	11.4	11.4	0.47	10.93
10/13/2009	11.4	11.4	1.04	10.36
10/14/2009	11.4	11.4	0.24	11.16
10/15/2009	11.4	11.4	0.29	11.11
10/16/2009	11.4	11.4	0.2	11.2
10/17/2009	11.4	11.4	0.07	11.33
10/18/2009	11.4	11.43	0.34	11.09
10/19/2009	11.4	11.4	0.02	11.38
10/20/2009	11.4	11.4	0.27	11.13

Horse ID= Uno (Sequence 2)

Date	Target Hay Intake (kg)	Hay Fed (kg)	Orts (kg)	Hay Intake (kg)
10/21/2009		11.36	0.81	10.55
10/22/2009	11.12 (2% BW)	11.36	0.29	11.07
10/23/2009	11.12	11.12	0.72	10.4
10/24/2009	11.12	11.15	0.43	10.72
10/25/2009	11.12	11.11	0.4	10.71
10/26/2009	11.12	11.12	0.11	11.01
10/27/2009	11.12	11.12	0.26	10.86
10/28/2009	11.12	11.12	0.04	11.08
10/29/2009	11.12	11.12	0.16	10.96
10/30/2009	11.12	11.12	0.07	11.05
10/31/2009	11.12	11.12	0.04	11.08
11/1/2009	11.12	11.12	0.1	11.02
11/2/2009	11.12	11.12	0.02	11.1
11/3/2009	11.12	11.12	0.1	11.02

Table 24: SAS program and raw data for determining effects of variables on acetic acid for EXPT2

```
Data batchvfa;
input horse $ sequence $ treatment $ period $ acetate;
Datalines;
ApolloPMAM  PM    1     59.09
Goose PMAM  PM    1     60.19
Vegas PMAM  PM    1     52.65
Uno   AMPM  AM    1     61.11
Diego AMPM  AM    1     56.54
Cal   AMPM  AM    1     68.22
ApolloPMAM  AM    2     61.63
Goose PMAM  AM    2     58.75
Vegas PMAM  AM    2     59.9
Uno   AMPM  PM    2     57.52
Diego AMPM  PM    2     52.75
Cal   AMPM  PM    2     60.24
;
Proc glm data = batchvfa;class horse sequence period treatment;
model acetate = sequence horse(sequence) treatment period /ss3;
test h=sequence e=horse(sequence);lsmeans period treatment sequence/
stderr pdiff;Title1 'PS2010_01 Batch Culture Acetate';Title2 'Molar %'
;run;
```

Table 25: SAS program and raw data for determining effects of variables on propionic acid for EXPT2

```
Data batchvfa;
input horse $ sequence $ treatment $ period $ propionate;
Datalines;
ApolloPM/AM PM    1     29.84
Goose PM/AM PM    1     25.25
Vegas PM/AM PM    1     15.71
Uno   AM/PM AM    1     17.27
Diego AM/PM AM    1     22.04
Cal   AM/PM AM    1     13.88
ApolloPM/AM AM    2     15.29
Goose PM/AM AM    2     27.38
Vegas PM/AM AM    2     20.3
Uno   AM/PM PM    2     18.36
Diego AM/PM PM    2     17.08
Cal   AM/PM PM    2     21.55
;Proc glm data = batchvfa;
class horse sequence period treatment;model propionate = sequence
horse(sequence) treatment period /ss3;test h=sequence
e=horse(sequence);lsmeans period treatment sequence/stderr pdiff;Title1
'PS2010_01 Batch Culture Propionate';Title2 ' Molar %';run;
```

Table 26: SAS program and raw data for determining effects of variables on butyric acid for EXPT2

```
Data batchvfa;
input horse $ sequence $ treatment $ period $ butyrate;
Datalines;

ApolloPM/AM PM    1     9.33
Goose PM/AM PM    1     10.69
Vegas PM/AM PM    1     17.48
Uno   AM/PM AM    1     15.88
Diego AM/PM AM    1     13.65
Cal   AM/PM AM    1     10.28
ApolloPM/AM AM    2     16.75
Goose PM/AM AM    2     8.8
Vegas PM/AM AM    2     17.73
Uno   AM/PM PM    2     15.53
Diego AM/PM PM    2     21.04
Cal   AM/PM PM    2     10.44
;
Proc glm data = batchvfa;
class horse sequence period treatment;
model butyrate = sequence horse(sequence) treatment period /ss3;
test h=sequence e=horse(sequence);
lsmeans period treatment sequence/stderr pdiff;
Title1 'PS2010_01 Batch Culture Butyrate';Title2 ' Molar % ';run;
```

Table 27: Plant carbohydrate fraction data for EXPT2

Period	Time	TNC	Starch	Mono	Gluc
1	800	15.34	3.49	5.56	2.36
1	1400	20.72	3.29	6.55	2.69
1	2100	19.32	3.56	6.11	2.31
2	800	11.84	2.95	3.84	2.133
2	1400	12.64	3.73	4.28	1.94
2	2100	14.82	2.84	4.82	2.66

Table 28: SAS program and data for determining effects of variables on total VFA for EXPT2

```
Title1 'PS2010_01 Batch Culture Total VFA';
Data FecalpH; input horse $ period treatment $ TotVFA;
cards;
Apollo2     am    80.12
Goose 2     am    80.83
Vegas 2     am    78.19
Uno   2     pm    85.82
Diego 2     pm    71.18
Cal   2     pm    69.35
Apollo1     pm    84.52
Goose 1     pm    84
Vegas 1     pm    73
Uno   1     am    94.14
Diego 1     am    99.37
Cal   1     am    85.26
;proc glm;class horse period treatment  ;
model TotVFA = horse period treatment;lsmeans treatment period horse
/stderr pdiff;run;
```

Table 29: SAS program and raw data for determining effects of variables on lactic acid for EXPT2

```
Data Lactate;
input horse $ period treatment $ sequence $   fecalLac;
Datalines;
Apollo1     PM    2     0.0025
Goose 1     PM    2     0.0039
Vegas 1     PM    2     0.0022
Uno'  1     AM    1     0.003
Diego'1     AM    1     0.004
Cal'  1     AM    1     0.0059
Apollo2     AM    2     0.00277
Goose 2     AM    2     0.0041
Vegas 2     AM    2     0.00458
Uno'  2     PM    1     0.016
Diego'2     PM    1     0.0084
Cal'  2     PM    1     0.016
;
Proc glm data = Lactate;class horse sequence period treatment;
model fecalLac = sequence horse(sequence) treatment period /ss3;
test h=sequence e=horse(sequence);lsmeans period treatment
sequence/stderr pdiff;Title1 'PS2010_01 Fecal Lactate';run;
```

Table 30: SAS program and raw data for determining effects of variables on fecal pH for EXPT2

```
Data pH;
input horse $ sequence $ period treatment $ fecalpH;
Datalines;
Apollo      pmam      2      am      7.69
Goose      pmam      2      am      6.94
Vegas      pmam      2      am      6.84
Uno'      ampm      2      pm      6.5
Diego'      ampm      2      pm      6.87
Cal'      ampm      2      pm      6.82
Apollo      pmam      1      pm      7.15
Goose      pmam      1      pm      7.3
Vegas      pmam      1      pm      7.28
Uno'      ampm      1      am      7.46
Diego'      ampm      1      am      6.7
Cal'      ampm      1      am      6.83
;Proc glm data = pH;
class horse sequence period treatment;
model fecalpH = sequence horse(sequence) treatment period /ss3;
test h=sequence e=horse(sequence);lsmeans period treatment
sequence/stderr pdiff;Title1'PS2010_01 Fecal pH';run;
```

Table 31: SAS program and raw data for NSC for EXPT2

```
Data pastnsc;
input pasture $ period $ treatment $ nsc;
Datalines;
1     1     am    18.08833333
2     1     pm    20.181
1     2     pm    13.72783333
2     2     am    12.24
;
Proc glm data = pastnsc;
class  pasture period treatment;
model nsc =  treatment period /ss3;
lsmeans treatment period /stderr;
Title1 'PS2010_01 Pasture NSC - Switch back analysis';
run;
```

Table 32: SAS program and raw data for determining effects of variables on BW for EXPT2

```
Data Bodywt;
input horse $ sequence $ period $ treatment $ BW;
Datalines;
Apollo2    1     PM    624
Goose 2    1     PM    538
Vegas 2    1     PM    598
Uno   1    1     AM    542
Diego 1    1     AM    584
Cal   1    1     AM    520
Apollo2    2     AM    616
Goose 2    2     AM    528
Vegas 2    2     AM    600
Uno   1    2     PM    548
Diego 1    2     PM    590
Cal   1    2     PM    526
;
Proc glm data = Bodywt;
class horse sequence period treatment;
model BW = sequence horse(sequence) period treatment /ss3;
test h=sequence e=horse(sequence);
lsmeans period treatment/stderr pdiff;
Title1 'PS2010_01 Body Weight';
Title2 'Day 12 - End of each period';
run;
```

Table 33: Grass heights (in) for P1, EXPT2

4	3.5	3	3.5	5.25
4.5	6.4	3	3.9	5
4.5	4.75	3.5	4	3.5
3.75	2	6.5	2.25	5.25
6.6	3.7	4	2.1	4.25
10	6	5	5.75	4
8.25	8.25	5.25	9.5	6
6.25	5.75	5.5	4.25	4.75
4	5.2	6.25	5.5	4.25
3	7.5	5	10	4.75
8.25	2.75	3.75	3.6	2.5
5.5	9.5	5	3.8	5.75
9.3	8.25	4.5	3.25	3.75
4.4	3.2	5	4.5	5.25
3.5	7	4	7.9	4
3	5.2	5.75	4.8	4.25
3.8	4.75	4.25	4.5	6.25
5.3	4	5.5	5.6	4
4.75	2.75	11.5	10.5	6.5
5.75	8.5	5	4.4	5
6.5	4	5.25	2.5	4.75
4.7	3.75	8.5	5.25	9.5
9.5	5.75	4	3.4	4.25
4.5	9	4.5	6.75	4.25
2.6	2.2	3.75	8.25	3.75
5.75	5.4	3.25	7	5.25
5.1	4.4	7.5	12.25	6.5
4.25	3	7	6.5	2.75
3	7.75	3.75	3	4
6.75	8.7	6.5	3	4.25
4.5	4.5	4.75	3	4
7.25	6	7	4	6.75
5.25	2.25			

Table 34: Grass heights (in) for P2, EXPT2

3.75	4	3	2.5	3.25
3.5	4.25	3.25	2	3
7.5	4.5	1.75	7.75	5.75
7.25	3.5	3.5	8	3.75
4.5	7	4.25	2.5	6.5
4.75	6.75	3.75	6.25	5
6.25	5	4.25	5.75	4
4.75	4	5.5	9.75	5.5
4	6.5	8.75	7.5	4
5	9.5	6.25	8.75	4.75
7.75	3.5	3.5	4.5	4
4	3.75	2.5	2.25	4.5
6.75	4.25	6.5	4.75	2.5
4.75	3.75	5	2.75	6.5
5	3.5	4.5	8	4.5
5.75	4.25	3	8	4
5.5	3	3.75	9.5	5.25
2.75	2.75	2.75	6.5	5.25
3	3.5	3.75	6.25	3
7.25	3	3.25	6	6.5
4.5	3.5	5.75	6.5	4.25
3	3.75	3.75	7	9.25
5.5	2.75	3.5	2.75	4
3.75	2.75	4.25	4	7.5
4.5	5.25	3.5	5.75	4
5	3	5.5	5.75	5.75
4.75	6.5	3	5.5	3.25
7.25	3.25	5.75	5.75	4.25
3.75	3.5	5.75	4.75	5.25
5	4.25	4.5	4.75	3.5
3.75	4	3.5	4.25	2.75
4.5	4.75	5	5.75	4.5
4.75				

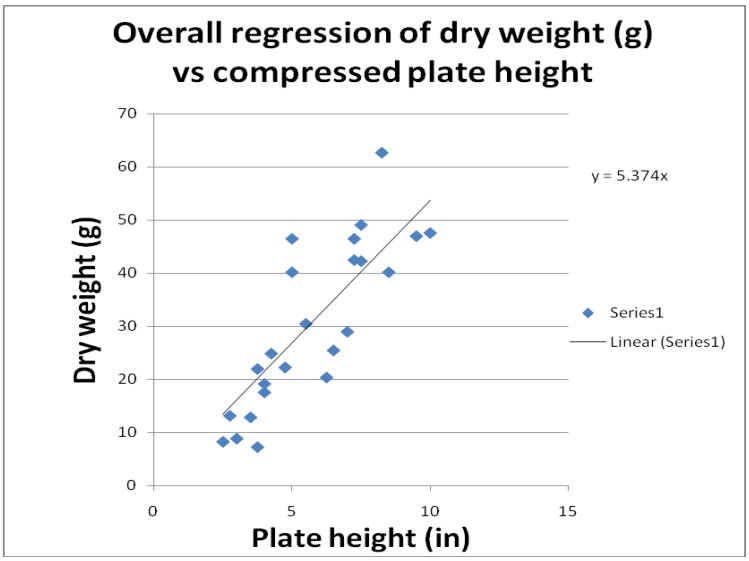

Figure 1: Overall regression of dry weight (g) vs compressed plate height (in)

Table 35: Methane data for P1, EXPT2

Period 1 Day 12

Bottle #	Sample	Rep	Substrate wt(g)	Methane(nmol/ml)
1	Horse1 **UNO**	1	1.02	14.98
2	Horse1	2	1.01	647.93
3	Horse1	3	1.01	355.05
4	Horse1	4	1.00	15.75
5	Horse2 **Cal**	1	1.01	39.99
6	Horse2	2	1.02	40.8
7	Horse2	3	1.01	124.48
8	Horse2	4	1.02	16.31
9	Horse3 **Diego***	1	1.00	19.44
10	Horse3	2	1.00	34.69
11	Horse3	3	1.00	44.39
12	Horse3	4	1.01	40.21
13	Horse4 **Apollo****	1	1.01	15.02
14	Horse4	2	1.02	28.71
15	Horse4	3	1.01	26.88
16	Horse4	4	1.01	52.6
17	Horse5 **Goose**	1	1.00	19.24
18	Horse5	2	1.02	15.72
19	Horse5	3	1.01	7.34
20	Horse5	4	1.02	15.5
21	Horse6 **Vegas*****	1	1.01	26.94
22	Horse6	2	1.00	188.6
23	Horse6	3	1.00	95.65
24	Horse6	4	1.00	19.4
25	Control, no feces	1	1.00	18.34
26	Control, no feces	2	1.01	19.8
27	Horse1 BLANK	1	--	27.99
28	Horse2 BLANK	1	--	112.38
29	Horse3 BLANK	1	--	61.58
30	Horse4 BLANK	1	--	29.42
31	Horse5 BLANK	1	--	22.1
32	Horse6 BLANK	1	--	482.36

Table 36: Methane data for P2, EXPT2

Period 2 Day 12				
Bottle #	Sample	Rep	Substrate wt(g)	Methane(nmol/ml)
1	Horse1Apollo*	1	1.01	30.96
2	Horse1	2	1.01	104.67
3	Horse1	3	1.02	11.04
4	Horse1	4	1.03	24.75
5	Horse2Goose	1	1.02	24.69
6	Horse2	2	1.00	1412.74
7	Horse2	3	1.01	1468.73
8	Horse2	4	1.03	25.905
9	Horse3Vegas	1	1.02	756.82
10	Horse3	2	1.02	33.1
11	Horse3	3	1.00	33.49
12	Horse3	4	1.01	722.33
13	Horse4Uno*	1	1.00	25.96
14	Horse4	2	1.02	30.35
15	Horse4	3	1.03	29.76
16	Horse4	4	1.02	26.76
17	Horse5Diego	1	1.01	24.52
18	Horse5	2	1.02	24.1
19	Horse5	3	1.01	77.60
20	Horse5	4	1.00	25.34
21	Horse6Cal	1	1.01	31.64
22	Horse6	2	1.00	36.77
23	Horse6	3	1.03	27.5
24	Horse6	4	1.02	26.58
25	Control, no feces	1	1.03	24.46
26	Control, no feces	2	1.02	29.82
27	Horse1 BLANK	1	-	34.33
28	Horse2 BLANK	1	-	534.74
29	Horse3 BLANK	1	-	138.34
30	Horse4 BLANK	1	-	818.295
31	Horse5 BLANK	1	-	34.48
32	Horse6 BLANK	1	-	23.58

Table 37: Plant carbohydrate fractions for EXPT2

Period	Time	Day	NSC	Starch	Mono	Gluc
1	800	1	16.227	3.538	6.485	2.753
1	1400	1	21.275	3.523	7.152	3.031
1	2000	1	16.823	3.457	5.954	2.175
1	800	2	14.445	3.442	4.637	1.966
1	1400	2	20.154	3.036	5.945	2.345
1	2000	2	21.816	3.652	6.271	2.434
2	800	1	11.454	3.862	4.002	1.528
2	1400	1	12.277	3.66	4.685	1.995
2	2000	1	14.201	2.957	4.757	2.469
2	800	2	12.269	2.873	4.175	2.234
2	1400	2	12.572	3.811	4.221	1.808
2	2000	2	16.102	2.93	5.75	2.918
2	800	3	11.794	2.1	3.333	2.637
2	1400	3	13.074	3.717	3.933	2.015
2	2000	3	14.141	2.62	3.966	2.597

Table 38: NSC averages for P1 and P2 for EXPT2

PS2010

Period 1	**NSC**			**Avg:**
8am	14.908	16.227	14.445	**15.19333**
2pm	21.521	21.275	20.154	**20.98333**
8pm	19.497	16.823	21.816	**19.37867**

Period 2				**Avg.**
8am	11.454	12.269	11.794	**11.839**
2pm	12.277	12.572	13.074	**12.641**
8pm	14.201	16.102	14.141	**14.81467**