The Pennsylvania State University The Graduate School College of Agriculture Sciences

# DIET AFFECTS GASTROINTESTINAL PERMEABILITY IN YEARLING

# **QUARTER HORSES**

A Thesis in Animal Science by

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# ABSTRACT

Increased gastrointestinal permeability in mammalian species has been shown to induce a variety of health consequences, and the capability to quantify and detect changes in gastrointestinal permeability is critical in understanding complex gastrointestinal disorders. In the horse specifically, the involvement of epithelial cell and tight junctional deterioration are hypothesized in the etiologies of laminitis induction, colic, and ulceration throughout the gastrointestinal tract. In this work, two forms of bovine colostrum were hypothesized to mitigate the predicted increase in gastrointestinal permeability induced by high starch, low forage diets in long Quarter Horse yearlings. In order to address this hypothesis, the optimization and validation of sugar absorption tests (SAT) in the horse as a measure of gastrointestinal permeability were performed. Nine Quarter Horse yearlings were utilized in a repeated Latin square design, which consisted of three 28 d periods. Each period contained one 14 d low concentrate high forage (LCHF) washout interval and one 14 d high concentrate low forage (HCLF) treatment interval. During the HCLF treatment intervals, yearlings were placed in one of three supplemental groups: whole bovine colostrum (WBC), concentrated bovine colostrum (CBC), or no supplement (NC), with each yearling receiving each supplement once throughout the study. Weekly fecal pH measurements were taken along with bi-weekly meal response measurements for both packed cell volume (PCV) and total plasma protein (TPP). At the conclusion of each two-week period a SAT was performed on each animal. Intricacies involving useful sugar marker dose, appropriate feeding protocol, urine collection and preservation, alditol acetate preparation for analysis, and protocols for gas chromatography with flame ionization detection of urine sugar markers have been

included as a part of validation of this technique in the horse. The SAT involved a sixteen-hour fast followed by administration of sugar markers via nasogastric tube to initiate a 24-hour total collection of urine. Fecal pH was lower on the HCLF  $(6.53 \pm 0.04)$ diet in comparison to the LCHF diet ( $6.95 \pm 0.04$ ) (P < 0.0001). Irrespective of diet, fecal pH was lower on day 14 than on day 7 of each interval ( $P \le 0.0001$ ). Fecal pH from day 7 to day 14 while horses were fed the LCHF diet decreased from  $7.08 \pm 0.05$  to  $6.81 \pm 0.05$ (P = 0.0003). Fecal pH from day 7 to day 14 while horses were fed the HCLF diet decreased from  $6.64 \pm 0.05$  to  $6.43 \pm 0.05$ . Packed cell volume decreased in response to concentrate meal to a greater extent on the HCLF diet (P < 0.0381), while meal response change of TPP was not affected by diet. Body temperature increased 0.304° F during the HCLF diet and 0.017° F during the LCHF diet when compared to an average measure during the LCHF intervals ( $P \le 0.0001$ ). The percent excretion of oral dose for sucrose, lactulose, mannitol, and sucralose was not different during the 24-hour urine collection for supplement treatments (P > 0.10). The data within the smaller 6-hour urine collections supports future investigation of CBC as a potential gastrointestinal health supplement. The percent excretion of oral dose of sucrose, lactulose, and mannitol when fed the HCLF diet indicate an increase in gastrointestinal permeability in comparison to intervals when fed the LCHF diet (P < 0.05). This study provides a preliminary evaluation of changes in equine gastrointestinal permeability due to diet and supports numerous studies that hypothesize high starch, low forage diets influence gastrointestinal health.

Key words: sugar absorption tests, high starch, nutrition, horse, restricted forage

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# LIST OF NON-STANDARD ABBREVIATIONS

BW	Body weight
CBC	Concentrated bovine colostrum supplement
СНО	Carbohydrate
<sup>51</sup> Cr-EDTA	<sup>51</sup> Chromium-labeled ethylenediaminetetraacetic acid
GC-FID	Gas chromatography with flame ionization detector
HCl	Hydrochloric acid
HCLF	High concentrate low forage diet
LCHF	Low concentrate high forage diet
LPS	Lipopolysaccharide
NC	No colostrum supplement
NSAID	Nonsteroidal anti-inflammatory drug
NSC	Non-structural carbohydrate
PCV	Packed cell volume
PEG	Polyethelene glycol
SAT	Sugar absorption test
TPP	Total plasma protein
<sup>99</sup> Tc-DTPA	Technectium 99m diethylenetriaminopentaacetate
VFA	Volatile fatty acid
WBC	Whole bovine colostrum supplement

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# **CHAPTER 1: REVIEW OF THE LITERATURE**

# Introduction and rationale

The epithelial barrier of the gastrointestinal tract is a complex and dynamic tissue, which collectively is the body's largest barrier to external pathogens. The selectively permeable gastrointestinal barrier is not only responsible for nutrient and water absorption, but is additionally critical in regulating immune status and symbiosis with the gut microbiota. Depending upon the specific anatomical location, the gastrointestinal barrier consists of mucus and cellular layers that function to regulate the many complex functions of absorption, secretion, regulation, protection, and growth within the barrier. The extensive microbial populations within the gastrointestinal tract are capable of utilizing carbohydrates (CHO) that are non-digestible via mammalian enzymes. From CHO fermentation, the microbial populations produce products, which provide energy, protein, and vitamins to both the horse and other microbes. The importance of the selective permeability of the gastrointestinal tract is highlighted in the etiology of gastrointestinal disease. Deterioration of both the epithelial cell and proteinaceous junctions of the barrier may lead to painful ulceration and non-selective absorption of lumen contents into the circulation (Mani et al., 2012). Certain products of microbial lysis and fermentation, such as lipopolysaccharide (LPS) and vasoactive amines have been associated with laminitis induction in the horse (Garner et al., 1975; Bailey et al., 2003). Prolonged administration of non-steroidal anti-inflammatory drugs, dietary induced changes in microbial fermentation, and physical insult, such as torsion of the anatomical structures, have been shown to increase the in vivo and in vitro permeability of the gastrointestinal tract (Garner et al., 1975; Bailey et al., 2003; O'Conner et al., 2004; Blikslager and Marshall, 2012). Diseases of the equine gastrointestinal tract are generally

multifactorial in nature, creating very complex interactions between each of these factors. Age, environment, genetics, nutrition, and a variety of other factors each play a role in determining animal health. The horse is fed to meet its individual nutrient and energy requirements, which is in contrast to many group fed livestock species. Applied precision feeding and the knowledge of appropriate feeding strategies allow for the mitigation of disease risk. For example, limit feeding of forages is known to induce gastric ulcers within horses (Murray, 1994) and high starch or fructan rich intakes potentially lead to colic and laminitis (Al Jassim and Andrews, 2009). As of yet, there remains to be a practical, on-farm equine diagnostic test, which allows for site-specific identification of gastrointestinal damage.

The sugar absorption test is a mixture of orally administered, mono- and disaccharides that are collectively utilized for site-specific identification of gastrointestinal permeability (Bjarnason et al., 1995). The appearance of these markers within the circulation or urine is quantified as representations of gastrointestinal permeability. These tests are widely utilized in the detection and monitoring of human gastrointestinal disease for conditions such as Irritable Bowel Syndrome, Celiac Disease Crohn's Disease, and colitis (Bjarnason et al., 1995; Arrieta et al., 2006).

Interestingly, bovine colostrum has been successfully implemented as a gastrointestinal health supplement, utilized for both gastrointestinal symptom mitigation and for the improvement of human athletic performance (Playford et al., 2000; Shing et al., 2009; Cairangzhuoma et al., 2013). Colostrum is the initial secretion post-partum from the mammary gland that provides vital initial nutrients, growth, and immune factors for proper health of the infant. In many mammalian species this substance serves as the

initial foundation for the infant's immune system, because immunoglobulins and other factors are passively absorbed by the gastrointestinal tract (Elizondo-Salazar and Heinrichs, 2008). The mechanisms via which colostrum mitigates gastrointestinal damage in other species has yet to be fully elucidated, however it is likely that a combination of growth factors, immunoglobulins, and other compounds aid in the protective function of this biological supplement.

In order to best manage and care for the horse, it is critical that digestive processes, absorptive mechanisms, and factors that impact gastrointestinal function in the horse be better understood. The use of a simple diagnostic test to identify changes in permeability in the gastrointestinal barrier, such as the sugar absorption test, creates many opportunities for the advancement of equine gastrointestinal health.

This review will provide a brief discussion of equine carbohydrate digestion, consequences upon gastrointestinal health of limit forage feeding and high starch feeding, techniques for measuring gastrointestinal permeability, and the potential benefits of supplementation with bovine colostrum.

# Carbohydrate digestion and absorption in the horse

Throughout history the horse has been exploited for its incredible athletic capabilities, initially as an integral tool of warfare and agriculture, yet today in developed nations we use the horse mainly for recreation. Anatomy of the horse's gastrointestinal tract is evolutionarily adapted for continuous, low energy density feeds, fitting the diet of wild horses on vast open plains. With domestication, the horse's lifestyle has been drastically modified to better conform to human conveniences. Horses are no longer consistently grazing on abundant fresh forages, but are often fed two concentrate meals per day and are fed limited forage. Caloric energy requirements are determined on a categorical basis dependent upon basal maintenance requirements and additional requirements for physical activity. Horses enduring very intensive exercise, such as those involved in racing or elite 3-day eventing, often require large quantities of energy dense feed, such as cereal grains, in order to satisfy their elevated energy requirements (NRC, 2007).

The ingestion of both concentrate and forages begins with the mastication of feeds at the broad, grinding surface of the tooth. Mechanical breakdown of feed, in combination with alimentary salivary secretions, which in the horse are initiated only by jaw movement, begin digestion. Feed then enters the stomach of the horse where it undergoes initial digestion. The small intestine is primarily responsible for enzymatic digestion and absorption of nutrients. Any remaining undigested products then enter into the hindgut, collectively the cecum and colon, for microbial fermentation. The hindgut is often compared to the rumen of the cow; a vat of symbiotic microbial fermentation that provides an energy source for the animal (Staniar and Maulfair, 2012). The rumen exists at the initial portion of the ruminant gastrointestinal tract, directly following the esophagus, in contrast to the hindgut of the horse, which follows the small intestine. This fundamental anatomical difference between ruminants and hindgut fermenters dictates different appropriate feeding strategies between these two groups of herbivores.

Protein and fat may be incorporated into an equine diet more effectively than that of a bovine diet. This is because the equine small intestine is capable of utilizing protein and fat prior to it potentially reaching the hindgut for fermentation or disruption of microbial populations. More concentrated energy ingredients, such as cereal concentrates and vegetable oil, have been incorporated into the equine diet as a functional and more practical solution to feeding the horse. In contrast to the ruminant, it is standard practice within equine husbandry to meal feed concentrates and forages to the horse. This is less likely to cause disruption within the equine gastrointestinal tract in comparison to the ruminant gastrointestinal tract due to fundamental anatomical differences discussed previously. The small intestine allows for extensive enzymatic digestion to occur prior to fermentation within the hindgut, this is in contrast to the rumen, which directly receives starch consumed by the cow. Dietary induced gastrointestinal diseases may arise when the gastrointestinal tract is without feed or substrate availability is abruptly modified. Limit feeding of forages to horses is a known procedure in inducing gastric ulceration (Murray, 1994). Acidosis within both the hindgut of the horse and the rumen can result from excessive, rapid fermentation of starch or fructans, which result in a low luminal pH. Ruminal acidosis is classified as a pH of less than 5.0 to 5.2, while subacute acidosis is less acidic and defined as 5.2 to 5.6. These conditions result in decreased production and can lead to laminitis in the cow (Nocek, 1997; Owens et al., 1998; Stone, 2004). In the horse, this decrease in luminal pH and resultant disruption of mucosal and epithelial linings may develop into more serious, even life threatening conditions such as laminitis and colic, similar to that which occurs in the dairy cow (Al Jassim and Andrews, 2009). Many major health concerns in both equine and livestock industries have the potential to be avoided if appropriate feeding management is implemented. As a herbivore, the horse's diet consists primarily of plant sources, as such an understanding of plant CHO composition and their behavior in the gastrointestinal tract is critical when formulating the equine diet.

# Carbohydrate fractionation

Carbohydrates serve as a main energy source for the horse, both from fresh and preserved forages and cereal grains. The maturity of the plant largely determines CHO composition and to a large extent digestibility of the plant. Physically effective fiber has been thoroughly investigated with importance to rumen function and is defined as physical characteristics that impact both consumption activities and rumen particle separation. Particle size is a primary constituent of the definition of physically effective fiber (Mertens, 1997). As a result, processing largely determines plant digestibility due to its impact on particle size and structural characteristics of both forages and concentrates. The impact of physically effective fiber has been investigated extensively with regard to the ruminant and its influence on productive parameters, but has yet to be implemented as a requirement in the equine diet (NRC, 2007; Staniar and Maulfair, 2012). Due to the similarities between the rumen and hindgut of the horse, physically effective fiber has the potential to impact equine digestive processes as well.

Carbohydrate fractionations are quantified through a variety of standardized analysis methods and provide a detailed map of the plant composition. Wet chemistry, near infrared reflectance, and in vitro digestion systems are each viable techniques of CHO evaluation. Each technique provides a slightly different interpretation of composition or predicted behavior within the gastrointestinal tract (NRC, 2007). The most precise and commonly utilized analysis involves wet chemistry and will thus be discussed further.

Plant CHO are primarily discussed with regard to plant structure or with regard to significance within animal nutrition. Structurally, the two major portions within the plant are the cell wall and cellular contents, within these broad divisions lies further

fractionation dependent upon molecular composition. Cellular contents consist of organic acids, sugars, starches, and fructans, while the cell wall consists of cellulose, hemicellulose, pectins, beta-glucans, and galactans (Hall, 2003). Classifications of plant CHO via wet chemistry include acid detergent fiber, neutral detergent fiber, non-starch polysaccharides, neutral detergent-soluble fiber, non-neutral detergent carbohydrates, and non-structural carbohydrate (NSC) (Hoffman et al., 2001; Hall, 2003). Plant fractionations included in the neutral detergent fraction include hemicellulose, cellulose, lingo-cellulose, and lignin. Aside from lignin, each of these fractions is fermentable via microbes. Lignin is non-digestible (Hoffman et al., 2001). The appropriate classification of NSC is frequently debated as a result of minor differences that occur as the result of wet chemistry analysis. The extraction process of low molecular weight CHO is performed utilizing either cold water or varying concentrations of ethanol (Hall, 2003). The important differentiation between water soluble CHO and ethanol soluble CHO is the inclusion of fructans. Extraction via cold water leads to organic acids, mono- and oligosaccharides, and various polysaccharides including fructans, pectic substances and dextrins (Hall, 2003). In comparison extraction utilizing ethanol will result in organic acids, mono- and oligosaccharides, however are less likely to include the polysaccharides listed in water soluble CHO. The degree of polysaccharide polymerization is thought to determine the solubility of fructans in ethanol, with concentrations of 80 to 90 percent ethanol increasing fructan solubility (Hall, 2003). For the purpose of this review, NSC is defined as starch content and water soluble CHO, thus NSC includes fructans.

Non-structural CHO content is frequently broadly associated with gastrointestinal implications within the horse. Enzymatic digestion of hexoses, disaccharides, certain

oligosaccharides and starches occurs within the small intestine of the horse.

Alternatively, the remaining sub-fractionations of NSC; resistant starches, galacto- and fructo-oligosaccharides, gums, mucilages, pectins, and algal polysaccharides, do not have the potential to be hydrolyzed within the small intestine of the horse and are subsequently rapidly fermented in the hindgut (Hoffman et al., 2001).

Starches and fructans have been implicated in equine diseases such as gastric ulceration, colic, and the initiation of laminitis (Garner et al., 1975; Bailey et al., 2003; O'Conner et al., 2004; Blikslager and Marshall, 2012). Understanding the digestive processes that occur within the gastrointestinal tract of the horse are of particular importance in understanding the disturbances they may initiate.

# Foregut digestion and absorption

Feed ingested by the horse is first mechanically digested via the grinding of the teeth and then chemical digestion is initiated via salivary amylases. Salivary excretions from the parotid gland in the horse are only produced as a result of mastication (Alexander, 1966). This connection to intake is important as saliva contains many factors, such as sodium, potassium, calcium, and phosphate, that are critical to buffering capacity within the stomach (Eckersall et al., 1985). The stomach of the horse is comprised of three different portions; the non-glandular fundic, glandular cardiac and pyloric regions. Hydrochloric acid (HCl), secreted from the parietal cells of the glandular regions of the stomach, functions to further digest the ingested feed. The stomach of the horse is unique in that acid secretion is continuous and cyclical regardless of feed ingestion (Campbell-Thompson and Merritt, 1987; Murray and Schusser, 1993). This relates to the anatomical evolution of the horse as a continuous grazer. The pH of the equine stomach ranges from

1.6 to 5.9 (Murray and Schusser, 1993; de Fombelle et al., 2003). The secretion of HCl in combination with infrequently fed, low forage diets or diets high in hydrolysable CHO are known to increase risk of gastric ulcers in horses (Murray, 1992; Nadeau et al., 2003a, b). The equine stomach harbors microbial species capable of producing volatile fatty acids (VFA) that additionally contribute to the risk for ulceration (de Fombelle et al., 2003; Nadeau et al., 2003a, b; Perkins et al., 2012). Three VFAs found at highest concentrations within the gastrointestinal tract of both ruminants and non-ruminants include acetic acid, propionic acid, and butyric acid. These three VFA have similar pKa values, while HCl and lactic acid are considered more acidic with lower pKa values (Argenzio et al., 1991). Specific VFA production is largely dependent upon diet and increases in the presence of hydrolysable CHO (Argenzio et al., 1974b).

The cellular structure of the equine stomach varies between the upper and lower divisions. The upper portion of the stomach, the fundic region, is comprised of a non-glandular stratified squamous epithelium. This region is most susceptible to ulceration because it does not possess the protective mucus layer present in the lower glandular portions of the stomach (Andrews and Nadeau, 1999). Gastric ulceration has been associated with both HCl and VFA concentrations within the stomach and will be discussed in further detail later in this review.

Upon leaving the gastric portion of the equine digestive tract, feed enters the duodenal portion of the small intestine. The small intestine is responsible for the enzymatic digestion and absorption of monosaccharides. Starch digestion begins with the hydrolysis of  $\alpha$ -1-4 glycosidic bonds by both pancreatic amylase and  $\alpha$ -amylase. This enzymatic action yields maltose and maltotriose, which are subsequently acted upon at

the brush border of the enterocytes via maltase, yielding glucose. Hydrolyzable carbohydrates must first be digested into monosaccharides in order to be absorbed by the enterocyte and utilized in biochemical processes. A variety of oligosaccharidases and disaccharidases exist at the brush border membrane (Freeman, 2012). The activity of brush border enzymes, maltase and sucrase, of horses consuming grain diets were not different from those maintained on pasture. Indicating that the upregulation of brush boarder enzymes does not occur when horses consume concentrate feeds (Dyer et al., 2002; Dyer et al., 2009).

Carbohydrate or monosaccharide transporters within the small intestine include SGLT-1 and GLUT-5, which exist on the apical membrane and GLUT-2, which is present on the basolateral membrane to transport monosaccharides out of the cell (Freeman, 2012). GLUT-5 transports fructose into the enterocyte via facilitated diffusion (Merediz et al., 2004; Freeman, 2012). Glucose is transported into the enterocyte via the sodium dependent transporter, SGLT-1 (Geor and Harris, 2007). The glucose then enters systemic circulation via the basolateral membrane glucose transporter, GLUT-2 (Hoffman et al., 2001; Salmon et al., 2002). SGLT-1 transporters have been shown to be upregulated 2-fold in the jejunum and 4- to 5-fold in the ileum in the presence of grain concentrates (Shirazi-Beechey, 2008; Dyer et al., 2009). Despite the evidence of upregulation of glucose transport, horses that are meal fed 2.0 grams of starch per kilogram bodyweight per meal have the potential to exceed the limits of enzymatic digestive capacity and monosaccharide transport in the small intestine (Kienzle, 1994). Small intestinal starch overload results in a modification of available substrate to the hindgut which may lead to microbial population shifts, acidotic changes, and damage to the epithelial barrier similar to that observed in subacute ruminal acidosis in cows (Nocek, 1997).

As a generalization, the primary function of the foregut is enzymatic digestion and the function of the hindgut is fermentation of non-hydrolysable CHO materials. Contrary to this generalization, the small intestine of the horse maintains microbial populations. de Fombelle et al. (2003), identified total anaerobic bacterial within the jejunum (8.7 log<sub>10</sub> c.f.u. per ml) and ileum (7.9 log<sub>10</sub> c.f.u. per ml) to be as great as those populations within the cecum (7.6 log<sub>10</sub> c.f.u. per ml), right ventral colon (8.1 log<sub>10</sub> c.f.u. per ml), and left dorsal colon (9.1 log<sub>10</sub> c.f.u. per ml). Total VFA concentrations are more reflective of the extensive microbial fermentation occurring within the hindgut: the jejunum (3.6 mmol/l), ileum (4.5 mmol/l), cecum (82.6 mmol/l), right ventral colon (89.1 mmol/l), and left dorsal colon (56.5 mmol/l) (de Fombelle et al., 2003). Enzymatic digestion is the primary function of the small intestine, however as evidenced by VFA concentrations quantified in the more distal portions of the small intestine there is fermentation occurring prior to the hindgut of the horse.

#### Hindgut digestion and absorption

The hindgut of the horse; the cecum, right ventral colon, left ventral colon, left dorsal colon, right dorsal colon, and small colon, functions very differently from that of the small intestine and is responsible for electrolyte, water, and VFA absorption. Sodium transport and osmotic pressure are thought to be the major regulatory mechanisms of net water movement in the large intestine (Argenzio et al., 1974a, b; Rakestraw and Hardy, 2012). The cecum and colon contain extensive fermentative microbial populations, whose VFA production vastly exceeds those in the stomach and small intestine (de Fombelle et al., 2003). Broad classifications of the microbial species are based on the substrates that they utilize as energy and nutrient sources; amylolytic, cellulolytic, lactic acid producing and utilizing bacteria, xylanolytic, and pectinolytic. The presence of various phyla within the hindgut is largely dependent on diet and substrate availability, as microbial species are opportunistic in nature (Medina et al., 2002).

The cecum is a 30 L blind sac sub-divided by taniae and haustra that aid in mixture of feed contents and microbial niches (Rakestraw and Hardy, 2012). The colon is a narrower structure than the cecum but collectively is capable of retaining 50 to 60 L of material (Rakestraw and Hardy, 2012). Each segment of the hindgut is thought to serve a mildly different function in regard to digestion. Various flexures and narrowings throughout the large intestine are thought to function in particle size retention similar to that which occurs in the rumen. Retaining larger particles for a longer period of time allows for further digestion. The cecocolic orifice, pelvic flexure, and transition from right dorsal colon to the transverse colon regulate rate of passage of varying particle sizes throughout the equine large intestine (Björnhag et al., 1984).

As a result of the differences in substrate availability within the various segments of the gastrointestinal tract, it would follow that the microbial populations within each segment of the hindgut are also different from one another. The metabolite, biochemical, and genetic profiles of microbial populations were quantified within the cecum, right dorsal colon, and feces of eight horses (Dougal et al., 2012). The various microbial profiles identified that the fecal contents of horses was similar to right dorsal colon contents, however the cecal contents were different from right dorsal colon and fecal profiles (Dougal et al., 2012). This gastrointestinal segment variation highlights differences in digestive function and with future work may be utilized to characterize subtle physiological differences. This work provides evidence that fecal samples may only be representative of the more distal portions of the hindgut. The syntrophic balance of the microbial population is dependent upon diet and additionally is varied from animal to animal. Animal to animal variation is not surprising due to genetic differences, with a specific regard to immune recognition and differing substrate availability provided by diet. This diverse and ever changing microbial variation within the equine hindgut has proved to make research on identifying and quantifying all of the equine hindgut species challenging. Additionally, while the presence of microbes can be identified with new sequencing technologies there is no way to match products of fermentation to specific species without complete sequencing and metabolic profiling.

Collectively, the hindgut microbial population exists as a syntrophic community, meaning that microbial species survival depends on the presence of other microbes and their products. Volatile fatty acids produced by individual bacterial species serve as an important nutrient source for both other microbial species and the horse. Dependent upon forage digestibility, up to 80% of the horse's energy needs have been shown to be met through a forage diet via the VFAs produced by the microbes (Glinsky et al., 1976; Vermorel et al., 1997). Horses fed a high forage to concentrate ratio diet, if any concentrates at all, will have minimal quantities of NSC that reach the hindgut due to the previously discussed enzymatic degradation within the small intestine (Arnold, 1982; Dyer et al., 2002, 2009). Those NSC that do reach the hindgut are fermented allowing for total tract starch digestion between 94 and 97% (Arnold, 1982). This complete total tract

digestibility indicates that NSC that escapes the small intestine are fermented in the hindgut as well as structural CHO (Arnold, 1982).

# Rate of passage

Rate of passage is a complex characteristic of equine digestion. Initial work done in 48 Shetland ponies evaluated the passage of a variety of gastrointestinal markers through the gastrointestinal tract (Argenzio et al., 1974a). Markers varied in size and were administered to the cecally and colonically cannulated horses during the middle of the initial morning meal and appearance at various locations of the gastrointestinal tract recorded. Polyethelene glycol (PEG) 4000 was utilized as a fluid marker throughout the tract and <sup>51</sup>Chromium-labeled ethylenediaminetetraacetic acid (<sup>51</sup>Cr-EDTA) as a differential fluid marker of the cecum and the colon. By 30 minutes the PEG-4000 liquid marker was in equal parts of the stomach, duodenum, jejunum, and ileum. An hour and a half post administration, approximately 25% of the liquid marker had reached the cecum with lesser amounts in the initial portions of the tract. By 2 and 4 hours post administration, the PEG-4000 liquid markers remained in the right ventral colon, until passing through the various portions of the colon (Argenzio et al., 1974a). When <sup>51</sup>Cr-EDTA was administered directly to the cecum it took approximately 12 hours for the majority of the marker to pass to the left ventral colon and another 24 hours to reach the right dorsal colon. At this 48 hour mark there was still liquid marker that remained in the cecum and colonic portions of the tract prior to the right dorsal colon. Larger particulate markers were also administered and seemed to follow a similar pattern as those identified for <sup>51</sup>Cr-EDTA through the hindgut. A final measure over the remaining 10 days indicated that it took 2 days for <sup>51</sup>Cr-EDTA to leave cecum entirely, and approximately 6

and 8 days for it to leave the ventral and dorsal colon, respectively. Larger particulate markers were maintained for longer periods of time than the fluid markers (Argenzio et al., 1974a). This highlights the differential strategies of the equine foregut and hindgut in digestion, particularly fermentative profiles, which will be differentiated in the upcoming section.

Smaller feed particles such as pelleted grains have been shown to pass through the cecum and into the colon much more rapidly than longer forage particles (Drogoul et al., 2000). The cecum possesses the lowest concentrations of both starch and lactate utilizing bacteria than any other region of the gastrointestinal tract of the horse (de Fombelle et al., 2003). As a result of both passage rate of smaller particles and bacterial populations, it has been suggested that the cecum plays a larger role in fermenting the structural components of CHO. In addition, when horses consumed 2.86 g starch per kg body weight in contrast to higher fiber diets, the addition of starch induced larger microbial changes within the colon microbial population than in the cecal populations (de Fombelle et al., 2003).

#### Fructan consumption

Fructans are polymers of fructose, storage CHO found in cool season grasses, produced at periods of high photosynthesis and stored for future use by the plant. Fructans can be found at highest concentrations in the spring during rapid growth of the plant. Fructan concentrations are particularly minimal in both summer and winter in conjunction with minimal growth of the plant (Pollock and Cairns, 1991). During periods of rapid plant growth when grasses are rich in fructan content (Hall, 2003; McIntosh, 2006), the horse is at risk for hindgut disruption due to its inability to enzymatically digest fructans. If consumed in high enough quantities, fructans have the potential to cause decreased hindgut pH similar to that of starch overload (van Eps and Pollitt, 2006; Millinovich et al., 2007). This concept of fructan-induced laminitis is currently a focus area within current research, however specific detail will not be included within this review (Johnson et al., 2013).

### *High starch meal feeding to exceed enzymatic capacity*

The extent of starch digestion within the small intestine is dependent on a variety of factors including rate of passage, extent of enzymatic capability, and intake (Julliand et al., 2006). A series of studies performed on ileally cannulated horses determined that precedul starch digestion is heavily dependent upon the processing of the cereal grain, the more processed a feed the higher pre-cecal digestibility. In comparison with a variety of cereal grains, oats were digested to the greatest extent. An important finding of the paper was that despite the variation in small intestinal degradation, the large intestine consistently compensated in fermenting all escaped NSC (Arnold, 1982). This same group continued on to quantify the amount of starch required to exceed the enzymatic digestive capacity of the small intestine (Potter et al., 1992). Following two experimental pilot studies, two ileally cannulated horses were fed a range of 0.1 to 5.2 kg of chopped corn per feeding twice a day. These meals resulted starch intake in a range of 0.2 to 5.5grams of starch per kilogram bodyweight per meal. As with their previous work, the total tract starch digestibility was approximately 97.0%, meaning that the large intestine fermented whatever NSC escaped the small intestine. According to their findings, prececal starch digestibility reached a maximum when fed at 3.5 to 4.0 grams of starch per kg bodyweight per meal (Potter et al., 1992). In comparison, Kienzle (1994) compiled a series of studies and found that at 2.0 g starch per kg body weight per meal there was a

40% reduction in digestibility across four commonly fed cereal grains. While these two studies provide a wide range of suggested limitations, they both conclude that there is a limitation to starch digestion within the small intestine. Inappropriate feeding strategies including limited feed availability and exceeding starch digestion within the small intestine play a role in several disease etiologies that are abundant throughout the industry.

# Prevalence of current nutritional health issues

#### Gastric and duodenal ulceration

Gastroduodenal ulceration is known to be a common issue throughout the equine industry regardless of discipline. Clinical symptoms associated with gastroduodenal ulceration are nondescript identifiers such as a lack of appetite, abdominal discomfort, and weight loss (Murray et al., 1989). The prevalence of gastric ulcers within the following equine populations includes: 70.9% in Thoroughbred broodmares (le Jeune et al., 2009) , 67.0% in endurance animals (Nieto et al., 2004), 72.2% in race horses (Lester et al., 2005), 58.0% in show jumpers and dressage animals (McClure et al., 1999), and 40.0% in western performance animals (Bertone, 2000). The definition of ulceration varied across the reported studies and does not include duodenal ulceration. Ulceration within the cardiac portion of the stomach and duodenum are more difficult to quantify via endoscopy and thus prevalence is challenging to quantify. It is assumed that an equine athlete experiencing discomfort as a result of ulceration will not perform at its highest capability.

# Colonic ulceration

In comparison to gastric and duodenal ulceration, less is known about the cause of colonic ulceration in the horse. Nonsteroidal anti-inflammatory (NSAID) administration

is a possible cause of ulceration and has been associated with right dorsal colitis. Symptoms include weight loss, diarrhea, hypovolemia, depression, and abdominal pain (Karcher et al., 1990). Ultrasound is a technique that has been utilized in evaluating damage within the dorsal colon, however the cost and practicality of this technique in the field has limited its utilization (Jones et al., 2003). Horses (n = 365) of various backgrounds were compared to 180 performance horses post-slaughter and examined for the presence and degree of both gastric and colonic ulceration. These two groups were selected to represent non-stressed and high-stressed animals. 87% of the presumably higher stress animals possessed gastric ulceration and 63% of the 180 sampled expressed colonic ulceration. In comparison, the larger population of non-stressed animals sampled 55% presented with gastric ulceration and 44% had developed colonic ulceration. While the non-stressed animals had a lower percentage than stressed animals, both groups possessed high percentages of ulceration (Pellegrini, 2005). Due to the nature of the study the cause of both gastric ulceration and colonic ulceration was unidentified, but leaves a clear identification as to an area that requires further investigation. It is possible that the some of the symptoms described in colitis case studies result as a disturbance in appropriate VFA absorption in the hindgut.

### Clinical acidosis: Carbohydrate induced laminitis

Unlike gastric ulcers, the complete mechanisms underlying laminar disruption in association with CHO overload and fructan consumption have yet to be fully elucidated. In 2000, the United States Department of Agriculture (USDA) conducted a national survey that extensively detailed lameness and perceived causes within the equine industry. The survey found that of the identified cases of laminitis, 7.4% were perceived to be initiated by grain overload and 45.6% due to grazing lush pasture. Additionally, at

the time of the survey only 2.1% of the population was reported to have been afflicted with laminitis within the past 12 month period (2000). The cases of laminitis reported do not include subclinical cases, as there are no symptoms exerted by the animal, however it is likely that a subclinical response could potentially limit performance ability and put the animal at a higher risk for gastrointestinal complications. One hypothesis of the onset of laminitis initiation is by either prolonged exposure to LPS or vasoactive amine concentration in the blood (Bailey et al., 2003). Laminitis induction models consist of starch overload, oligofructosaccharide, and black walnut administration (Garner et al., 1975; Milinovich et al., 2007; Belknap, 2010). The exact mechanistic link between the effects of alimentary administrations to changes in laminar structure has yet to be completely elucidated. A time-course evaluation of histopathologic equine small intestinal and cecal samples during laminitis induction revealed extensive mucosal damage. 72 hours following CHO overload, there was disruption of the proteinaceous tight junctions, increases in intercellular spaces, vacuole formation, and swelling of mitochondria within the epithelial barrier of cecal samples (Krueger et al., 1986). Although not quantified in these studies, these changes are consistent with the LPS and lactate translocation hypothesized to occur in laminitis induction models (Krueger et al., 1986; Masty and Stradley, 1991). In vitro models of incubation with corn starch and inulin, a type of fructan, have shown to increase permeability in conditions that mimic those that occur in in vivo laminitis induction models (Weiss et al., 2000; Bailey et al., 2003).

## Subclinical acidosis

The impact of subclinical acidosis in both high producing ruminants and nonruminants is of concern and particularly difficult to identify due to the lack of specific physiological symptoms. Outside of a research setting it is difficult to quantify the impact of subclinical cases, however several techniques provide an approximation of hindgut physiology. The increased permeability of the gastrointestinal tract and thus entry of LPS into circulation are associated with laminitis and colic (Al Jassim and Andrews, 2009). The subclinical form of equine hindgut acidosis however is often not recognized due to the lack of specific external clinical signs that horses present in association with this disease process. In the dairy cow, subacute acidosis is defined by a ruminal pH of 5.2 to 5.6 (Owens et al., 1998). Such broad definitions do not exist in the horse, likely due to a lack of access to cecal and colonic samples. Research on subclinical acidosis in the horse has been limited to studies of cannulated animals or sacrifice studies resulting in a relatively limited data set to evaluate. Fecal pH and fecal VFA quantification serve as indirect representations of hindgut acidity. There is weak supporting evidence that these animals develop stereotypies such as crib-biting, stall walking, and general irritability as a result of discomfort (Willard et al., 1977). A positive significant association between cecal lactate concentration and wood chewing was found in horses fed high starch diets  $(P \le 0.01)$  (Willard et al., 1977). This association with negative behaviors supports the possibility of a higher incidence of subclinical acidosis than currently reported.

The impact of high fiber diets in comparison to a high starch diets on pH throughout the entire gastrointestinal tract has been investigated (de Fombelle et al., 2003). A fiber pellet and straw diet (528 g/100 kg BW NDF per day and 142 g/100 kg BW starch per day) was compared to a cereal grain (type unspecified) and meadow hay diet (428 g/100 kg BW NDF per day and 233 g/100 kg BW starch per day). The pH of the stomach, duodenum, and cecum were higher on the grain and meadow hay diet, while

pH in the small colon was lower on the grain and meadow hay diet (P < 0.05). The range of pH across both diets within the small intestine and large colon was 5.8 to 7.4 (de Fombelle et al., 2003).

The effects of high grain diets on fecal parameters of racehorses utilized in the Australian Thoroughbred horseracing industry were investigated (Richards et al., 2006). Seventy-two trainers were surveyed and it was determined the average total grain fed to each horse was 7.33 kg/day, with a maximum total grain fed per day of 13.20 kg. Due to the nature of this study it is difficult to identify the exact dietary composition, however oats, commercially premixed diets, corn and barley were the four most commonly fed grains. Following the analysis of fecal pH and fecal volatile fatty acid (VFA) production, this group concluded that hindgut acidosis was occurring in 27% of the horses utilized in the Australian Thoroughbred industry. Interestingly, the authors did not specifically define subclinical acidosis, but rather drew conclusions based on the increased propionate and hypothesis that cellulolytic bacteria do not survive at pH below 6.2 (Richards et al., 2006).

In contrast, six Thoroughbred fillies were monitored for fecal pH and populations of *Lactobacillus* and *Streptococcal* species during a transition from fresh forage on pasture to dried forage, high concentrate diets that were within the limits of starch digestion (van den Berg et al., 2013). Dietary concentrate to forage ratio was increased over a 13-day period, with a maximum starch intake of 3.3 g dry matter per kilogram body weight per meal on day 13. Days 14 to 16 horses were abruptly returned to pasture. Significant increases in both microbial species were detected when horses were fed concentrate (P < 0.001) and decreased when returned to pasture (P < 0.001). However, despite increases in starch utilizing bacteria, fecal pH values during concentrate supplementation never fell below the initial baseline value recorded while on pasture and thus no associations to changes in pH were observed (van den Berg et al., 2013). This observation supports the theory that fiber inclusion within the diet may suppress the negative impact of high NSC consumption on microbial populations as proposed by (Medina et al., 2002). It is additionally possible that the change in fecal pH was not observed, as the horses were not maintained on the high starch intake levels for a long enough period. Subclinical changes within the gastrointestinal tract are very difficult to identify and quantify, particularly outside of a research setting.

# Mechanisms of acidotic disturbances within the equine gastrointestinal tract

Feeding NSC, via cereal grains, to the horse are an effective and viable option to increase the energy density of the diet, however careful consideration must be implemented. For the purposes of this review, the acidotic cascade is defined as the resultant increase in lactic acid, decrease in pH, changes in microbial population, and endotoxin release from gram-negative bacteria cell walls that occur as a result of NSC appearance. Concentrate feeding has been associated with gastric ulcers in the horse and more notably the acidotic cascade linked to starch excess and fructans consumption. The mechanisms involved in these disease processes are similar in nature due to the negative impact of excessive acidity on epithelial barrier. Luminal pH, lactate concentrations, LPS translocation, and VFA production can be utilized as indirect indications of epithelial induced acidotic changes within the horse's gastrointestinal tract. A direct measurement of gastrointestinal tissues is impractical in most diagnostic and research situations. Factors such as individual horse variability, starch source, type of feed processing, dietary forage, and other management factors all play a role in the risk management of this disease process. The acidotic cascade that results in decreased luminal pH is well understood through ruminant research and is thought to be very similar in the horse. There remains a need to better understand the etiology of this disease process in the horse and its implications on the performance and overall health of the animal.

### *Hindgut acidotic cascade*

The processes that occur during carbohydrate overload induced acidosis of the equine hindgut are very similar to ruminal acidosis (de Fombelle et al., 2003). The decrease in cellulolytic activity results in a decrease in the proportion of acetate, while the acidophilic increase leads to an increase in the proportion of propionate and lactate These changes are initiated and supported by a change in dietary substrate availability (Nocek, 1997; de Fombelle et al., 2001).

As early as 1965, the condition of acidosis has been recognized to have been initiated by feeds high in starch and sugar (Dunlop and Hammond, 1965). Due to the accessibility to the rumen via cannulation, acidosis has been thoroughly modeled in ruminant systems and less so via non-ruminant cecal and colonic cannulations. When a large, starch or fructan dense concentrate diet is consumed, the amount of NSC entering the fermentative organ changes the substrate availability drastically from that observed on a high fiber diet. Certain opportunistic gram-positive bacteria preferentially utilize these substrates more than gram-negative, lactate-utilizing and cellulolytic species. Cattle fed ad libitum entirely concentrate diets had microbial populations which consisted of 82 to 95 percent of starch hydrolyzing bacteria and no cellulolytic bacteria (Slyter et al., 1970). The addition of starch to the hindgut consistently results in significant increases in the proportion of starch utilizing bacteria (Garner et al., 1978; Julliand et al., 2001; Bailey et al., 2003). This shift in microbial populations leads to the accumulation of D-lactic acid, which as mentioned earlier has a lower pKa value than acetate, propionate, and butyrate. D-lactic acid is produced primarily by microbial fermentation and is not metabolized as rapidly as L-lactic acid, because D-lactate is produced primarily from microbes within the lumen it serves as an indication of increased permeability (Ewaschuk et al., 2005). The increase in production and limited clearance combine to exacerbate the decline in pH associated with the acidotic cascade. Many studies do not differentiate between the two isomers, however other metabolic processes within the body may produce L-lactate thus complicating results (Ewaschuk et al., 2005; Al Jassim and Andrews, 2009).

With specific focus on the etiology of acidosis, two main species that produce lactic acid include *Streptococcus* and several *Lactobacillus species*. *Streptococcus bovis*, a gram-positive bacterium, is capable of digesting starch into maltose as a result of its capability to produce amylase. *S. bovis* has been shown to maintain proliferation capabilities at pH values much lower than those of other ruminal species (Russell et al., 1979). *Streptococcus* are not as acid tolerant as *Lactobacillus* species, however they have a growth capacity that allows them to double in less than 12 minutes (McAllister et al., 1990). This capacity for rapid proliferation of primarily *S. bovis* leads to the further decline of pH as a result of lactic acid production. Once pH drops below 5.3 the growth of *Streptococci* are reduced, yet *Lactobacilli*, another lactate producing species, flourish in the acidic environment and continue the pervasive pH decline (Nocek, 1997).

Prior to the overgrowth of gram-positive lactate producing bacteria, several types of bacteria are important for the prevention of the acidotic cascade. *Megasphera elsdenii* 

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and *Selenomonas ruminatium* are gram-negative lactate-utilizing microbes, however *S*. *ruminatium* does not preferentially degrade lactate like *M. elsdenii*. *M. elsdenii* has been investigated in in vitro systems to ferment 60 to 80 percent of D/L-lactate (Counotte et al., 1981). Together these species play a large role in removing lactic acid from the rumen and hindgut. *M. elsdenii* species stops growing when the pH drops below 5.6 and *S*. *ruminatium* at 5.1 and the lack of ruminal presence leads to an accumulation of lactic acid (Russell et al., 1979). The lysis of these two lactate-utilizing species from ruminal interactions plays a large role in the microbial cascade involved in acidosis.

The lysis of bacteria within the rumen and hindgut in relation to changes in pH leads to an important differentiation in bacterial classification. Gram-positive bacteria, such as *S. bovis* and *Lactobacillus*, possess a thick peptidoglycan outer membrane, which is not present in gram-negative species. Conversely, gram-negative, *M. elsdenii* and *S. ruminatium*, species have LPS, also known as endotoxin, within their outer membrane (Elin and Wolff, 1976). Lipopolysaccharide is an antigenic glycolipid which consists of three polysaccharide portions and the bioactive, lipid A portion (Mani et al., 2012). Monomeric LPS molecules vary in size (10 to 20 kDa) and may form large micelle of up to 1,000 kDa (Mani et al., 2012). Increased LPS appearance in the plasma is utilized in the same fashion as D-lactate for detection of gastrointestinal damage. Tight junctions are the primary route of translocation of LPS in an inflammatory state (Mani et al., 2012). Lipopolysaccharide is typically cleared from the bloodstream via the liver, however if appearance in plasma exceeds liver clearance rate then systemic disruption occurs (Hackett and Hassel, 2008). Elevated blood LPS has been linked to starch and fructan overload, but has not yet been described as a direct cause of laminitis (Sprouse et al.

1987; Bailey et al., 2003). Lipopolysaccharide blood concentration is not consistent throughout all laminitis cases and the mechanisms via which it causes laminitis are still being evaluated, however this variability may largely be due to the rapid clearance of LPS from plasma (Sprouse et al., 1987). Sprouse et al. (1987) identified two large peaks during laminitis induction within plasma of afflicted horses. The 16-hour interval indicates a unique clearance of LPS known as a Schwartzman phenomenon. Additionally, the authors hypothesized that LPS may be cleared from equine circulation in less than 4 hours (Sprouse et al., 1987). This was the first work to directly quantify LPS levels within the plasma of laminitic horses, however several previous authors proposed LPS as an explanation for circulatory collapse in laminitis induced animals (Garner et al., 1975, 1977). With specific concern towards laminitis, LPS commonly found in the lumen of the gastrointestinal tract is hypothesized to induce irreversible, lamellar changes within the hoof (Garner et al., 1975).

# Consequences of acidosis: Gastric

The anatomy of the equine stomach has adapted over millions of years to continuous grazing and entry of fibrous grasses. As such, the equine stomach continuously secretes HCl (Campbell-Thompson and Merritt, 1987), this becomes a problem in the horse that does not have access to forages to maintain stomach fill. Forage particle sizes are separated within the equine stomach and provide a stratification of pH that decreases nearest the pyloric region. In the absence of forages, the non-glandular mucosa may be exposed to very low pH contents due to the absence of feed buffer (Argenzio and Southworth, 1975). This tissue is more sensitive to acid contact as it is not protected by a mucosal buffer and is the most prevalent site for ulceration in the horse (Murray, 1994).
The withdrawal of forage is known to induce gastric ulcers in horses. A feeding schedule containing 12 and 24-hour alternating periods of hay availability and removal over a 7 day period consistently induces gastric ulceration in the horse (Murray, 1994). This pattern of feed deprivation is now the common technique used in ulcer induction models (Murray and Eichorn, 1996; O'Conner et al., 2004; Morrissey et al., 2010). The percentage of pyloric measurements below a pH of 2.0 in fasted and hay fed horses were 76% and 30%, respectively over a 24-hour period (P = 0.05). Median gastric pH of fasted horses in the pyloric antrum, 1.55, was significantly lower than hay fed horses at a pH of 3.1 (P = 0.007) (Murray and Schusser, 1993). Ulceration was not quantified on this study, however it is evidence that the gastric pH is buffered in the presence of feedstuffs and likely mitigates ulceration.

Variations in substrate availability are known to influence products of fermentation; additionally these products have been implicated in ulceration pathways (Argenzio et al., 1991). The impact of an all bromegrass hay diet or an alfalfa hay and grain diet upon gastric pH, VFA production, lactate production, and degree of ulceration were evaluated (Nadeau et al., 2000). Hours 1, 2, 3, and 5 post-feeding, horses on the alfalfa and grain diet had higher acetic acid production than those maintained on bromegrass hay alone ( $P \le 0.05$ ). Hours 1 through 6 post feeding horses, on the alfalfa and grain diet had higher propionic acid production than those maintained on bromegrass hay alone ( $P \le 0.05$ ). No differences in diet were observed in valeric or isobutyric acid concentrations and butyric acid was higher at the first sample post feeding on the bromegrass diet. Gastric pH samples collected from 2 to 5 hours after feeding were higher in alfalfa and grain fed animals than in bromegrass fed animals (P = 0.01). Despite

higher concentrations of acetate and propionate on the alfalfa and grain diet, bromegrass fed animals exhibited a greater number and severity of non-glandular ulcerations (P = 0.05) (Nadeau et al., 2000). The authors attributed the difference in ulceration to the differing calcium and protein content of the two diets. The inclusion of calcium has been shown to mitigate gastric secretion, this theory was further supported by the decrease in pH 5 hours following the meal (Behar et al., 1977). Protein content has been previously shown to improve buffering capacity in ruminants (Haaland et al., 1982).

Feed deprivation and hydrolysable carbohydrates both function to decrease the luminal pH of the gastric contents. In the lack of presence of feedstuffs, continuously secreted HCl, and increased VFA and lactic acid concentrations derived from the fermentation of hydrolysable carbohydrates within the stomach collectively lead to a suppression of pH. The absorption of VFA is dependent upon the luminal acidity, as passive absorption is determined by the dissociation state of the VFA. In a lower pH environment, VFAs are more easily absorbed into the cells of the gastrointestinal tract (Bugaut, 1987). The mechanisms via which VFA cause cellular damage were elucidated by Argenzio et al. (1996) in a porcine in vitro model. It has been proposed that upon absorption into the cell, the VFA functions to acidify the inner contents of the gastrointestinal cell. This acidity then leads to disregulation at the sodium pump, initiating osmotic imbalance.

The non-glandular squamous epithelium of the upper portion of the equine stomach is most susceptible to ulceration because it lacks protection from a mucus barrier such as that present in the glandular portions of the stomach. This more vulnerable squamous mucosa is comprised of four main cellular layers; stratum corneum, stratum transitionale, stratum spinosum, and deepest, the stratum basale (Argenzio, 1999; Andrews et al., 2005). This cellular layer is held together via tight junctions that prevent the passage of sodium and HCl (Andrews et al., 2005). Deeper within the squamous mucosa are sodium transporters and buffering compounds. Once the stratum corneum layer is disrupted, there is little to prevent further damage from occurring within these upper gastric tissues.

In contrast, a bicarbonate-rich mucosal barrier lining the glandular epithelium of the stomach, phospholipid in nature serves to protect the epithelial cells from hydrophilic HCl (Hills and Kirwood, 1992; Ethell et al., 2000), cellular swelling, and apoptosis (Argenzio and Eisemann, 1996). Damage that occurs within the glandular mucosa of the stomach is generally attributed to NSAID medications that disrupt the circulatory flow to the mucosal barrier, thus impacting prostaglandin production (Andrews et al., 2005). Due to the focus of this review on dietary factors, NSAID disruption will not be discussed at length.

### *Consequences to acidosis: Small intestine*

Damage to the small intestine of the horse has been extensively investigated in both ischemia reperfusion models and NSAID administration models (Marshall and Blikslager, 2011). The direct impact of high starch and fructan models has been limitedly investigated (Masty and Stradley, 1991). The Paneth cell, a cell located in the crypts of the small intestine, secretes antimicrobial factors and functions to modulate microbial growth in the gastrointestinal tract. A CHO induced laminitis model (Laminitis Inducing Ration) identified the structural changes that occur within Paneth cells following carbohydrate overload. This is indicative of the emptying of lysozymal components into the lumen of the gastrointestinal tract (Masty and Stradley, 1991). The tissues and timecourse of the healing process of the jejunum is different from that of the large intestine as evidenced by differences in transepithelial electrical resistance (TER) recovery time (Tomlinson et al., 2004; Matyjaszek et al., 2009; Marshall and Blikslager, 2011). Transepithelial electrical resistance is an in vitro evaluation of the permeability of epithelial barriers as maintained by tight junction proteins. Intact barriers maintain differential compartments between the lumen and basolateral surface of the epithelial cells, allowing for an electrical difference to occur (Blume et al., 2009). Three main stages of recovery within the small intestine include contraction of the villi, mucosal repair and epithelial restitution, and finally the closure of paracellular spaces at the tight junction (Blikslager et al., 2007). The tight junction serves as the main control of barrier function (Blikslager et al., 2007). In the event of a compromised epithelial barrier, such as those induced by weakened tight junction proteins, the horse is at risk for gastrointestinal disease and further complications.

# Consequences to acidosis: Cecum and large intestine

Changes in cecal mucosa over a 72-hour period post laminitis induction with corn starch were evaluated (Krueger et al., 1986). At 24 and 32 hours post induction, cecal epithelium appeared to be swollen and the initiation of epithelial degeneration appeared via scanning electron microscopy. Further, cellular disruption and sloughing was observed at 48 and 72 hours. At 72 hours post administration there was disruption of the tight junctions, with visible wide intercellular spaces indicating hydrophobic changes and disruption, with some samples containing no recognizable junctions. Similarly, swollen mitochondria and extensive vacuole formation occurred at 72-hour sampling (Krueger et al., 1986). This study is the only of its kind in which direct samples were removed for histological examination of the equine hindgut during laminitis models.

More recently, Weiss et al. (1998) observed a transient increase in technectium TC99m diethylenetriaminopentaacetate (<sup>99m</sup>Tc-DTPA), an indicator of gastrointestinal permeability, following corn starch induction of laminitis. Two 8-hour periods beginning at 4 and 20 hours post laminitis induction with CHO were evaluated via 8-hour urine pools and hourly blood sampling. Urinary excretion of <sup>99m</sup>Tc-DTPA increased from 2.45% of oral dose in control animals to 16.67% in animals between 4 and 12 hours post administration and returned to values similar to baseline (3.57%) at 20 to 28 hours post administration. Blood radioactivity followed urinary excretion (Weiss et al., 1998). These brief changes in permeability differ drastically from the structural changes observed in the previously mentioned study, however may be indicative of further changes within the small intestine in association with those indicated by Masty and Stradley in 1991 (Krueger et al., 1986). Another discrepancy of this work is that no differences in plasma LPS were observed in association with the increase in permeability. The authors attribute this to the insensitivity and false identification of endotoxin presence of the Limulus amebocyte assay utilized (Weiss et al., 1998).

Structural changes within the rumen have been identified in association with decreased ruminal pH. The impact of subacute ruminal acidosis on rumen papillae structure was investigated in four non-lactating dairy cows (Steele et al., 2011). The cows were switched to a high grain diet (35% chopped hay 65% mixed grain) designed to decrease ruminal pH. The pH was lowest during the first week of the high grain diet and reached minimum values of 5.3, mean pH during the initial week was 5.8. Organization

disruption and thickening of the stratified squamous epithelium was thought to result in increased permeability, although this characteristic was not measured directly. Cellular migration was increased as well. This study provides support that luminal pH values characterized as subacute acidosis, 5.6 to 5.2, have the potential to cause damage to the enterocyte epithelial lining (Steele et al., 2011).

## Gastrointestinal barrier and permeability

The gastrointestinal tract of any mammalian system serves as one of the body's largest protective barrier systems against external pathogens. Selective capacity of the gastrointestinal tract epithelial lining is critical in maintaining health of the animal; all essential nutrients must be absorbed while simultaneously preventing the absorption of pathogens and other harmful molecules. There are a variety of protective and homeostatic characteristics involved in the gastrointestinal barrier.

#### *Properties of the intact gastrointestinal barrier*

The epithelial barrier that lines the gastrointestinal tract serves many functions in both the physical and immunological health of the animal. The surface area of the gastrointestinal tract acts as the largest protective barrier from outside pathogens than any other component of the body. The barrier in its entirety consists of the mucosal layer, epithelium, and lamina propria (Keita and Söderholm, 2010). The mucosal layer separates the lumen of the gastrointestinal tract from the epithelial cells and includes a water layer, the glycocalyx, and the mucus layer. Secretory immunoglobulin A plays a major role in this section of the barrier in bacterial defense (Keita and Söderholm, 2010). In addition to the IgA function at the level of the mucosal layer, the epithelial cells also possess extensive secretory antimicrobial peptide functions. This aids in the success of the innate immune system in clearing any potential pathogens from the system prior to the initiation of an infection (Keita and Söderholm, 2010). The cells within the gastrointestinal tract are organized into multiple cellular layers to allow for continuous regeneration. Each of the compartments of the gastrointestinal tract has a slightly different cellular layout that allows it to optimize the particular function of that section. For example differences in the glandular and non-glandular sections of the stomach or the extensive villi within the small intestine necessary for nutrient absorption. The integrity of this protective barrier is critical in maintaining the health of the individual.

The epithelial barrier consists of multiple cell types, the most abundant are the absorptive enterocytes with mast cells, Paneth cells, and goblet cells intermixed dependent upon anatomical location. Epithelial cells allow for passive diffusion of lipid soluble and discrete hydrophilic compounds, while slightly larger hydrophilic compounds (600 Da) may utilize the tight junction for passage into circulation. Antigen stimulating molecules are typically too large to pass through either of this routes in an intact barrier (Keita and Söderholm, 2010). The epithelial layer of the gastrointestinal tract is constantly replenished with new cells that derive from the pluripotent stem cells located in the crypt, this process occurs in both the small intestine and the colon (Bjerknes and Cheng, 1999; Pakkanen and Aalto, 1997; 2001). The epithelial cells of the mouse are completely replaced within 3-5 days from stem cell to villus tip (Bjerknes and Cheng, 1999).

There are several groups of proteins that function to maintain the selectively permeable epithelial layer. Three major groupings of proteins observed at the interface between cells include the tight junction proteins, adherens junction and the innermost desosomes (Farquhar and Palade, 1963). The main function of the tight junction relates to

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its location between the cells, as it plays the largest role in maintaining the regulation of paracellular transfer. Tight junctions are primarily composed of a group of membrane proteins called claudins. Currently twenty-seven specific claudin genes have been identified, each associated with a particular anatomical location (Günzel and Yu, 2013). Various claudin proteins have been measured as an indication of changes in tight junction status (Arrieta et al., 2006). Intermediately located, the adherens junctions have a wider variety of functions from epithelial growth regulation to polar interactions across proteins. Supportive strength to the tight junction is provided by the desmosome proteins (Marchiando et al., 2010). It appears the specific protein actin may play an extensive role in maintaining polarity of the cells through various locations and pathways throughout these junctions (Sheff et al., 2002). The complex interactions between cell type, protein junction, and chemical characteristics all form the epithelial barrier, the extensiveness of gastrointestinal disease impacts a variety of these components. This complexity of interactions makes it difficult to identify the exact mechanisms of failure. Various tests are utilized in human clinical diagnostics to provide a gross estimation of gastrointestinal permeability, or leakiness, which is indicative of failure of protective capacity.

# Measuring gastrointestinal permeability

Molecules within the gastrointestinal tract may be transferred from the lumen to the circulation via two main modes of action; transcellular and paracellular transport. Transfer of molecules through enterocytes is referred to as transcellular transport and occurs via active carrier-mediated absorption, endocytosis, passive diffusion, or aqueous pores (Keita and Söderholm, 2010). Paracellular transport, or movement in between cells, of hydrophilic molecules occurs across the proteins that serve to connect the various cells of the epithelial barrier (Watson et al., 2001). Each of these transport mechanisms play an important role in selectively absorbing nutrients and water while simultaneously preventing toxic and antigenic substances from entering the circulation.

The premise of intestinal permeability testing is based upon the appearance of administered molecules in the circulation as an indication of permeability of the gastrointestinal barrier to various sized markers. There are several qualities that are desirable to formulate the quintessential gastrointestinal permeability marker they include: non-toxic, non-metabolizable, not typically found as a portion of the diet, not endogenously produced, with rapid renal clearance (Davies, 1998).

The first utilization of this technique was by Menzies in 1974 utilizing oligosaccharides. Various test models include PEG, <sup>51</sup>Cr-EDTA, <sup>99m</sup>Tc-DTPA, LPS and D-lactate appearance in plasma, tight junction proteins in plasma, and differential sugar absorption tests (SAT) (Davin et al., 1988; Weiss et al., 1998; Sun et al., 2001; Watson et al., 2001; Frias et al., 2012). Sucrose, <sup>51</sup>Cr-EDTA, and <sup>99m</sup>Tc-DTPA markers have been utilized both in vitro and in vivo to evaluate gastrointestinal permeability in the horse (Weiss et al., 1998, 2000; O'Conner et al., 2004; Escala et al., 2006; Hewetson et al., 2011, 2012).

The implication of parasite infestation, specifically cyathostome larvae, was investigated utilizing <sup>51</sup>Cr-EDTA as a permeability marker. Cyathostome infection significantly increased percent excretion of <sup>51</sup>Cr-EDTA from baseline ( $3.95 \pm 0.66$ percent excretion) to infected animals 46 days post infection ( $11.66 \pm 6.71$  percent excretion) (Escala et al., 2006). The standard deviation for the later measurement is much larger than control and day 22 of infection, possibly indicating a wider degree of variation in parasite load. <sup>99m</sup>Tc-DTPA was utilized in an alimentary CHO laminitis induction model to evaluate mucosal permeability. Ponies developed Obel grade-1 or grade-2 by 30 hours. Percent excretion of  $^{99m}$ Tc-DTPA increased from control animals at 2.45% (range 1.73 – 3.16%) of administered dose to a mean of 16.67% (range 13.78 – 19.32%) administered dose at 4 to 12 hours post administration for laminitic animals (Weiss et al., 1998). Radioactive labels such as those listed above allow for easy quantification in biological fluids, however are of concern due to their radioactive characteristics. While radioactive techniques are employed when necessary in veterinary hospitals, this characteristic severely limits the use of such markers in on-farm diagnostics and research settings outside of veterinary facilities.

There is no ideal permeability marker available. Comparisons of applicability between SAT, PEG, and radioactive labels have been investigated in humans and results indicate similar excretion patterns between them (Anderson et al., 2004; van Wijck et al., 2012). Following the development of sucralose, a non-fermentable disaccharide, more research investigating the whole gut is being done with SATs.

### Sugar absorption tests

Common sugars utilized in SAT include sucrose, lactulose, rhamnose, mannitol, and sucralose. Sucralose is a newer compound and has not been used as extensively as the others listed. These markers are non-invasive, inexpensive, and quantifiable in urine and plasma, thus allowing for an effective model of gastrointestinal permeability evaluation. However, SAT are not without flaws and interpretation of excretion can be difficult. Rate of passage, differences in gastrointestinal surface area, excretion rate, fermentation rate, and other factors complicate the site-specific determination of gastrointestinal permeability. Of the most commonly implemented sugar markers, only the monosaccharides mannitol and rhamnose are capable of passing through smaller pores (4 to 5 Å) at the luminal surface. In contrast, the larger disaccharides are less affected by water flux and villi length because they are only capable of absorption deeper within the crypt where pore size is increased (10 to 20 Å or larger). The absorption of these molecules increases when tight junctions and epithelium are damaged (Arrieta et al., 2006).

Sucrose is a disaccharide that is degraded within the initial segment of the small intestine by sucrase, forming glucose and fructose, thus the appearance of whole sucrose in the urine is indicative of gastric permeability (Sutherland et al., 1994; Kızıltaş et al., 1998; O'Conner et al., 2004; Buddington et al., 2006; Hewetson et al., 2006; D'Arcy-Moskwa et al., 2012). Mannitol and rhamnose are both monosaccharides and typically one of these is utilized, not both. There is no clear difference as to which of these disaccharides provides a better marker of gastric and small intestinal permeability. Purified rhamnose is more expensive than mannitol.

Lactulose, a fermentable disaccharide, is most commonly administered as a gastric and small intestinal marker. It is often reported in a ratio to either rhamnose or mannitol, although the usefulness of this ratio is questioned in the literature. The fermentative capacity of the stomach and small intestine of the horse may negatively impact the usefulness of these sugars in the horse, although the rate at which these sugars are fermented has not been investigated. The rate of fermentation and factors that influence monosaccharide absorption lead to the complex interpretation of lactulose: mannitol. In the rat, mannitol has been shown to be absorbed through both passive diffusion (absorption during neutral water flux) and influenced by solvent drag as

changed by osmolarity of the gastrointestinal contents (Krugliak et al., 1994). This concept is an important consideration for the interpretation of this monosaccharide as it may be more influenced by osmolality within the gastrointestinal tract than other disaccharides due to it's smaller size.

Sucralose is a chlorinated form of sucrose and is not degraded at any point throughout the gastrointestinal tract and serves as an indication of whole gut permeability (Smecuol et al., 2001; Suenaert et al, 2003; Anderson et al., 2004). Sucralose is the main component of the artificial sweetener Splenda®, the lack of degradation, both enzymatic and fermentative result in its low calorie properties (Grotz and Munro, 2009). The premise behind the SAT is very simple, however when considering the application of any test within complex biological systems the interpretation of data may become challenging.

The derivitization processes and analytical techniques employed to quantify carbohydrates in biological samples are numerous and variable. Gas chromatography often requires a much more extensive derivitization process than that for liquid chromatography, due to the stabilization necessary for volatilization of molecules onto the gas column (Ruiz-Matute et al., 2011). While gas chromatography provides very reliable results, the lengthy derivitization process is often avoided if possible. Various forms of chromatography and analytical detectors ranging in sensitivity function to isolate and quantify specific compounds. High-performance liquid chromatographytandem mass spectrometry (Rao et al., 2011), high-performance anion-exchange chromatography with a pulsed amperometric detector (Wijtten et al., 2011), ion-exchange chromatography with mass spectrometry (van Wijck et al., 2012), and gas chromatography with flame ionization detector (Suenaert et al., 2003) have all been employed for the quantification of sugar permeability markers. The most precise techniques are those that utilize mass spectrometry, however cost and availability of equipment may be inhibitory in many situations. Additionally, not all of the equipment listed can easily determine all four sugar markers at the same time and often analyze lactulose and a monosaccharide only. Gas chromatography with flame ionization detection allows for the analysis of sucrose, lactulose, mannitol, and sucralose in the same run.

#### Sucrose absorption tests in the horse

To date, sucrose has been the only sugar utilized in the investigation of permeability testing in the horse, with a focus on gastric ulceration quantification. The appearance of sucrose in both blood and urine has been employed in testing for gastric ulcers on three occasions (O'Conner et al., 2004; Hewetson et al., 2006; D'Arcy-Moskwa et al., 2012). O'Conner et al. (2004) implemented ulcer induction via feed deprivation and found that the horses developed ulcers ranging from an ulcer score of 0 to 3 all within the non-glandular portion of the stomach. A score of zero was indicative of intact mucosal epithelium and a score of three indicated ulceration through the mucosal layer (O'Conner et al., 2004). The animals were each administered 454 g of sucrose dissolved in water via nasogastric tube and urine and blood samples were collected at 2 and 4 hours post administration. Urine was collected via urinary catheter and total mass excreted was calculated via high performance liquid chromatography with pulsed amperometric detection and adjusted based on urine volume. Urinary sucrose excretion collected 2 hours post administration of sucrose significantly differentiated ulcer scores of greater than 1 to less than 1. Additionally, ulceration scores of 2 and 3 could be differentiated from ulceration scores of less than 1, however the sucrose test was not capable of differentiating ulcers scores 2 from 3. Urine samples collected 4 hours post administration detected significant differences between scores of 3 and  $\leq$  2, as well as 1 from  $\leq$  1. This suggests that the test is applicable for use over a range of time periods. The specificity for detection of moderate ulceration was 83% and 90% for severe ulceration. It should be noted that during this study blood samples were additionally collected at 2 and 4 hours post sucrose administration, however were not analyzed due to lack of sensitivity in techniques at the time of publication (O'Conner et al., 2004).

Hewetson et al. (2006) evaluated the usefulness of serum samples to detect oral administration of sucrose in ulcer identification. High performance liquid chromatography with mass spectrometer was utilized and validated. Ulcers were induced in horses using feed deprivation and ulcer scores between 0 and 3 developed. Peak serum sucrose concentrations appeared for all ulcer grades 1 through 3 45 minutes post sucrose administration and were significantly correlated to severity (P = 0.001). Further, the authors proposed that sucrose may be a more effective tool in estimating gastric ulceration than endoscopy due to underestimation during the procedure of both severity and depth of gastric ulcers as supported by Andrews et al. (2002). The usefulness of utilizing serum parameters in field diagnostics has potential for advantages over urinary sampling and endoscopy if further validated in the horse. Currently laboratory equipment and preparation samples are expensive and difficult to conduct, of further benefit would be a much simpler analysis of marker concentrations within biological samples (Hewetson et al., 2006).

More recently the techniques for quantification of sucrose in equine serum have been further validated utilizing liquid chromatography equipped with mass spectrometer and implemented in equine models (D'Arcy-Moskwa et al., 2011, 2012). Ulcer induction in the most current study was induced via various NSAID treatments and horses were maintained on lucerne hay, with gastric permeability to sucrose (0.05 g/kg body weight) evaluated on days 0 and 13 of treatment. Serum parameters were measured 15, 30, 45, 60, 90, 120, and 240 minutes post sucrose administration. Only the group treated with the non-selective COX inhibitor, phenylbutazone, was found to be induce a significant difference (P = 0.001) in gastric permeability to sucrose, other animals treated with meloxicam showed no difference between day 0 and 13. Meloxicam is a selective NSAID inhibitor that preferentially targets the COX2 enzyme, allowing it to induce less side effects (Beretta et al., 2005). The authors attributed the lack of significance within other treatments to study design, minimal subjects (n = 5 per treatment), and weak ulcer induction. The use of gastroscopic evaluation of each group did not detect a difference (P = 0.657) and contradicts the differences detected via sucrose permeability (D'Arcy-Moskwa et al., 2012). As a result, this article was in agreeance with Hewetson et al. (2006) regarding the potential capability of sucrose to indicate ultrastructural damage that may be caused prior to ulceration such as that associated with NSAID (Davies, 1998). While these studies have shown that the sucrose permeability test is functional in the horse for predicting gastric ulceration, the analyses and instrumentation required to analyze samples have prevented it from being widely used for diagnostic purposes.

### Interpreting results

As a brief overview, the sugar absorption test typically involves the following sugars: sucrose, lactulose, rhamnose or mannitol, and sucralose. Sucrose is enzymatically

degraded upon entering the duodenum and thus serves as a gastroduodenal marker. Lactulose is utilized in combination with either rhamnose or mannitol as an indication of permeability through the small intestine. Sucralose is not fermented nor enzymatically degraded and serves as a whole gut permeability marker. It is important to highlight that lactulose and sucralose do not specifically identify small intestinal or large intestinal damage. The utilization of a combination of markers is necessary to evaluate anatomical sections of permeability (Meddings and Gibbons, 1998; Arrieta et al., 2006).

Sucrose is perhaps the easiest of the sugar markers to interpret due to the brief amount of relative time liquid markers are retained within the stomach relative to the rest of the gastrointestinal tract. As described previously, this marker has been implemented in gastric ulcer detection in horses (O'Conner et al., 2004; Hewetson et al., 2006; D'Arcy-Moskwa et al., 2011, 2012).

Lactulose and a companion monosaccharide, mannitol or rhamnose, are typically utilized in combination in order to evaluate gastric and small intestinal permeability. Lactulose is fermented via microbial populations, primarily in the large intestine. Lactulose is believed to not be influenced by luminal drag, as the pores through which lactulose is absorbed are located at the base of small intestinal villi (Arrieta et al., 2006). This is in contrast to smaller pores located at the apical tip of villi through which mannitol or rhamnose is absorbed (Arrieta et al., 2006). Due to these differences, monosaccharides are thought to provide an approximate estimate of small intestinal surface area. Thus an increase in the ratio of percent excretion of disaccharide to monosaccharide is either indicative of increased paracellular absorption or a decrease in small intestinal surface area (Arrieta et al., 2006). Sucralose is not fermented by microbes nor is it enzymatically degraded at any point throughout the digestive tract. Additionally, it behaves similarly to the other sugar markers routinely utilized in renal excretion (Meddings and Gibbons, 1998). Less frequently the ratio of sucralose to the other sugars is utilized (Meddings and Gibbons, 1998; McOmber et al., 2010). The utilization of these additional ratios is intended to provide a better understanding of specific anatomical location, but due to a host of factors that may influence sugar excretion it is difficult to draw specific conclusions.

The importance of duration of total urine collection duration is critical in interpreting the urine excretion data and has been studied more extensively in human clinical work (Camilleri et al., 2010; McOmber et al., 2010; Rao et al., 2011). Two common time intervals of urine collection duration in human studies include 0 to 5 hours as small intestinal permeability and a total 24-hour urine collection for whole gut examination. The importance of collecting pooled urine is important in identifying approximate timing of marker excretion in the different anatomical portions of the gastrointestinal tract. Whole tract SATs are novel in the horse there is still a general lack of knowledge regarding how factors such as passage rate, microbial fermentation, impact the validity of the test (Arrieta et al., 2006; Van Weyenberg et al., 2006).

There are a variety of pre- and post-mucosal factors that are of concern with regard to sugar absorption tests. In the presence of microbial overgrowth however, the interpretation of this marker may be confounded (Arrieta et al., 2006). Additionally, it is debated how quickly the lactulose enters and is degraded within the cecum and colon (Camilleri et al., 2009). To further complicate the interpretation of foregut permeability the ratio of lactulose to either rhamnose or mannitol is also difficult to interpret.

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Lactulose to rhamnose ratio has been shown to be increased by post-mucosal fluid loading (intravenous injection of saline) in rats (Hallemeesch et al., 2000), however 5 hour lactulose and rhamnose urinary excretion were not changed with the intravenous administration of 2 liters of fluid in healthy human volunteers (Parviainen et al., 2005). There are many factors that may potentially influence the percent excretion of sugar markers. It is of importance that results from both individual studies and across study comparisons be interpreted with caution.

### Bovine colostrum: potential benefits to oral supplementation

#### **Biological properties**

The biological function of bovine colostrum is to provide the newborn calf with energy, nutrients, antimicrobial secretory immunoglobulins, and multiple immune factors that are absorbed through pinocytosis (Hurley and Theil, 2011). These maternal immunoglobulins allow the calf to develop an early immune defense. The passive transfer of these components is critical to the calf's survival, as there is no immune transfer from the maternal blood supply to the calf in utero. The role of insulin-like growth factor on calf gastrointestinal development is a major focus of research, although it is acknowledged that colostrum additionally impacts digestive enzyme activity and other gastrointestinal hormones (Blum and Hammon, 2000). Bovine colostrum consists of an extensive variety of growth factors, immune components, and hormones that all play a role in gastrointestinal growth and development (Pakkanen and Aalto, 1997; Rawal et al., 2008). Bovine colostrum has been implemented as a successful gastrointestinal health supplement in human, mouse, and porcine under a variety of disease and insult models (Playford et al., 2000; Rawal et al., 2008). Additionally, bovine colostrum has also been reported to improve exercise performance in human athletes (Shing et al., 2009). Due to the complexity of components within colostrum it is very difficult to precisely identify single components that may be playing a role in improving gastrointestinal health and is likely to be a combination of multiple factors. The biological origin of colostrum has the potential to impact the content of important factors (Hurley and Theil, 2011). This raises concern for consistency of effectiveness as a gastrointestinal health supplement across batches of colostrum if utilized as a commercial product.

Bovine colostrum contains immunoglobulins, growth factors, and other hormones that each potentially influence interactions with microbiota and cellular proliferation of the gastrointestinal tract (Playford et al., 2000). Immunoglobulins G, A, and M are found within bovine colostrum, each with specific anti-viral, anti-bacterial, or anti-fungal functions (Rawal et al., 2008; Stelwagen et al., 2009). Immunoglobulin G (IgG) comprises the majority of immunoglobulin content in bovine colostrum and as a result is typically the characteristic used to evaluate the quality of colostrum (Stelwagen et al., 2009). The general function of immunoglobulins is to bind to specific antigens and present them to the functional cells of the immune system.

In addition to immunoglobulins, various growth factors may influence gastrointestinal healing and cellular turnover. An in vitro study performed on a human epithelial cell line indicates that the growth-promoting activity of bovine colostrum is likely primarily modulated by insulin-like growth factor-1 (IGF-1) and transforming growth factor- $\beta$  (TGF- $\beta$ ), but other factors could not be eliminated (Purup et al., 2007). Multiple stages of lactation were evaluated and found the growth promoting activity of lactation products were highest at day 1 postpartum and interestingly the week before

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drying off. Differences were also seen in day of lactation for IGF-1, TGF- $\beta$ 1 and TGF- $\beta$ 2 content (Purup et al., 2007). This variability emphasizes the importance of day of collection of colostrum to ensure consistency of the product.

## Supplementation in immunocompromised patients

As an antimicrobial supplement, the immunoglobulin content in bovine colostrum lends itself well to support the health of immune suppressed patients. HIV patients frequently suffer from life threatening diarrhea. Lactobin, a preparation of bovine colostrum containing 4.9 g IgG, 1.5 g IgA, and 0.5 g IgM, per dose were administered orally in solution once daily to 25 HIV-positive males. Twenty days of oral supplementation of immunoglobulin concentrate produced 36% of complete symptom remission and 22% partial symptom remission following treatment. The remaining 39% of subjects showed little to no improvement (Plettenberg et al., 1993). The variability of response in this paper is thought to be due to variation in etiology of HIV cases and organisms causing insult. Following an increased dose, one of the initially nonresponsive patients went into full remission, while the others remained non-responsive (Plettenberg et al., 1993). A more recent set of studies showed improved quality of life and a reduction of other physical symptoms following bovine colostrum supplementation (Florén et al., 2006; Kaducu et al., 2011). Duration and dose of supplementation may play a role in the effectiveness of bovine colostrum as an HIV supplement.

Rotavirus is a main cause of diarrhea in pediatric patients and poses serious health concerns with relation to dehydration. Increased intestinal permeability occurs during infection with rotavirus and damage at the tight junction has been observed (Beau et al., 2007; Dickman et al., 2000; Obert et al., 2000; Uhnoo et al., 1990). Various bovine colostrum treatments have been investigated as a tool to mitigate the associated gastroenteritis symptoms. In animal husbandry it is not uncommon to vaccinate the mother to ensure the offspring will obtain certain antibodies through colostral passive transfer. Hyperimmune bovine colostrum (HBC) is the initial fluid produced by cows, which were vaccinated against a particular target virus. This process allows for a specific antibody to be in higher concentration and thus provide the infant stronger defenses against target viruses. Patients treated with HBC showed a more rapid recovery from rotavirus than those supplemented with ordinary colostrum (Mitra et al., 1995). A more recent study addressed the protective capacity of bovine colostrum in rotavirus mouse models and found the importance of supplementation within 24-hours of viral inoculation to largely influence intensity of diarrheal symptoms (Inagaki et al., 2013).

# Supplementation in ulceration models

Non-steroidal anti-inflammatory drugs (NSAIDs) are a popular oral medication for many disease states, ranging from arthritis to cardiovascular disease. NSAIDs function to inhibit an enzyme involved in the inflammatory cascade, cyclooxygenase 2. However chronic NSAID administration often leads to deterioration and ulceration of the gastrointestinal tract. Bovine colostrum supplementation has been evaluated in a variety of disease states as a modulator of increased gastrointestinal damage and leakiness including those investigating NSAID induced damage (Playford et al., 2000). The impact of bovine colostrum supplementation on gastric and small intestinal injury in rat models was evaluated. Colostrum proved to display a dose dependent protective capacity of gastric damage. Additionally, colostrum supplementation mitigated the impact of reduced villus height within the small intestine significantly (Playford et al., 1999). Due to the successfulness of bovine colostrum in the rat model, the same lab conducted a follow up study in human subjects. Lactulose and rhamnose oral sugar markers were utilized to measure permeability. Supplementation with 125ml of bovine colostrum three times daily for 7 days resulted in 3-fold reduced permeability in comparison to indomethacin-induced control (Playford et al., 2001). A 2005 study measured the gastrointestinal permeabilities of 12 rats via 51-chromium ethylene diaminetetraacetic acid (<sup>51</sup>Cr-EDTA) following a challenge with diclofenac to increase damage. It was found that 7 days of supplementation with 5% bovine colostrum solution reduced permeability 2 fold from that of non-supplemented animals (Kim et al., 2005). The several growth factors present in bovine colostrum are likely the factors that initiate further proliferation of epithelium in the injured areas.

# Supplementation in heat stress induced gastrointestinal permeability models Intensive physical exercise has the potential to influence blood distribution

throughout the body in order to supply peripheral tissues with oxygen and nutrients during exercise. This preferential shift of blood away from the gastrointestinal tract has the potential to initiate bacterial translocation and immune response (van Wijck et al., 2012b). Gastrointestinal disturbance is known to impact athletic performance (Shing et al., 2009). Increased gastrointestinal permeability is observed in exercise induced heat stress models (Marchbank et al., 2011). Recovery of gastrointestinal tract permeability for high intensity athletes is critical to maintain top performance, and bovine colostrum is currently sold as a human neutraceutical for this audience (Marchbank et al., 2011). Extensive research has been done in the area of human athletic performance, yet due to the variation in defined baseline measurements, length of dose, and amount and quality of supplement the results are not conclusive across supplemental treatments as reviewed by Shing et al. (2009). The athletic performance parameters influenced by bovine colostrum supplementation are outside of the scope of this review and thus influence on exercise induced gastrointestinal permeability is highlighted. Gastrointestinal permeability was evaluated in 12 human participants following a supplementation period of 14 d with bovine colostrum. Participants endured a 20 min exercise challenge at 80% maximum oxygen consumption. Intestinal permeability increased 2.5-fold in the control group and was mitigated by approximately 80% following bovine colostrum supplementation (P <0.01) (Marchbank et al., 2011). The impact of bovine colostrum supplementation on athletic performance parameters has been more thoroughly investigated than its impact upon exercise induced gastrointestinal permeability during colostrum supplementation. It is possible that increased performance parameters are a result of improved barrier function; however further research is necessary to make this conclusion.

# **Summary**

The gastrointestinal barrier serves to maintain the healthy condition of all mammals as a complex integration of nutrient and water absorption and immunological and physical protection. The escape of immunogenic compounds into the blood stream due to inappropriate barrier function has the potential to create a systemic inflammatory response. The integrity of the enterocytes and their junctions is largely hypothesized in the etiologies of many human disease states from irritable bowel syndrome to Crohn's Disease (Dastych et al., 2008). Currently, no field diagnostic tests are employed to detect the condition of the equine gastrointestinal barrier. In veterinary hospital settings histological examination, surface oximetry (tissue oxygenation), fluorescein dye, ultrasonography, and luminal pressure are utilized as assessment tools (Blikslager and Marshall, 2012). The impact of subclinical acidosis due to high starch diets and the acidotic state they create within the hindgut is largely unknown. A better identification of the risk of high starch diets will largely benefit the athletic performance horse industry. Additionally, the employment of SATs in the horse may provide great insight into current major disease states such as equine metabolic disease, acidosis linked laminitis, or colic recovery or allow for the evaluation of dietary treatments, such as bovine colostrum.

# Hypothesis and objectives

It was hypothesized that bovine colostrum fed to Quarter Horse yearlings would mitigate the predicted increase in gastrointestinal permeability by the high concentrate low forage diet. The objective of this study was to quantify and describe the pattern of urinary excretion of four orally administered sugar markers in Quarter Horse yearlings fed diets formulated to impact gastrointestinal permeability. Additionally, fecal pH and microbial populations and meal-response blood parameters will be utilized to support the subclinical changes expected.

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# **CHAPTER 2:** The Impact of Diet and Colostrum Supplementation on Gastrointestinal Permeability

# Abstract

The selective absorptive capacity of the gastrointestinal tract is critical in both nutrient and water absorption. It is equally important in preventing antigenic compounds from crossing into the circulation. In this study, two forms of bovine colostrum were hypothesized to mitigate the increase in gastrointestinal permeability induced by high starch, low forage diets in Quarter Horse yearlings. To quantify changes in gastrointestinal permeability, the optimization and validation of sugar absorption tests (SAT) in the horse were performed. Supporting indirect variables were also measured in support of dietary induced changes in permeability. Nine yearlings were used in a replicated Latin square, consisting of three 28 d periods. Each period consisted of one 14 d low concentrate high forage (LCHF) washout interval and one 14 d high concentrate low forage (HCLF) treatment interval. During the HCLF intervals only, yearlings were placed in one of three treatment groups; whole bovine colostrum (WBC) (70 g topdressed on concentrate twice daily), concentrated bovine colostrum (CBC) (70 g topdressed on concentrate twice daily), or no colostrum supplementation (NC). At the conclusion of both the 14 d LCHF washout and HCLF treatment interval, a sugar absorption test (SAT) was performed on each horse. Each SAT consisted of a 24-hour urine collection to quantify excretion of administered sugar markers. Fecal pH was lower on the HCLF (6.53  $\pm$  0.04) diet in comparison to the LCHF diet (6.95  $\pm$  0.04) (P < 0.0001). Irrespective of diet, fecal pH was lower on day 14 than on day 7 of each interval  $(P \le 0.0001)$ . Response of PCV to concentrate meal decreased to a greater extent on the HCLF diet (P < 0.0381), while meal response change of TPP was not affected by diet.

Degree change in body temperature from washout average was higher when yearlings were fed the HCLF diet (P < 0.0001). The percent excretion of oral dose for sucrose, lactulose, mannitol, and sucralose was not different during the 24-hour urine collection for supplement treatments (P > 0.10). The data within the smaller 6-hour urine collections supports future investigation of CBC as a potential gastrointestinal health supplement. The percent excretion of oral dose of sucrose, lactulose, and mannitol when fed the HCLF diet indicate an increase in gastrointestinal permeability in comparison to intervals when fed the LCHF diet (P < 0.05). Limit feeding of forages, meal feeding of starch rich concentrates, or a combination of both may subtly influence gastrointestinal mucosa and overall permeability in the horse. Subclinical gastrointestinal disturbances will likely negatively impact nutrient and water absorption, and increase the risk of clinically relevant gastrointestinal disease. Further application of the sugar absorption test in the equine research has the potential to be informative in a variety of equine research areas.

Key words: gastrointestinal permeability, sugar absorption tests, horse, bovine colostrum, nutrition

# Introduction

The gastrointestinal barrier serves as a complex protective barrier from external pathogens, consisting of absorptive, secretory, and immune cells connected via proteinaceous tight junctions. A non-compromised gastrointestinal barrier functions to absorb nutrients and water and maintain immune homeostasis in order to maintain the health of the horse. Adequate identification of ulceration, deregulation of epithelial cell organization at the epithelial barrier, throughout the gastrointestinal tract is of major concern throughout the equine industry (Andrews et al., 2002). Deterioration of proteinaceous tight junctions or epithelial cells allows the passage of larger molecules to cross from the lumen of the gastrointestinal tract into circulation. These larger molecules have the potential to initiate an immunogenic response (Natividad and Verdu, 2012). Compounds of particular interest in the horse include endotoxins and vasoactive amines, both of which exist in a normal equine gastrointestinal tract. Endotoxins are derived from the cellular structure of gram-negative bacteria and become free structures upon the lysis (Mani et al., 2012). Vasoactive amines are products of both gram negative and grampositive fermentation. Endotoxins and amines, which inappropriately enter the circulation via a leaky gut, have been hypothesized to induce site-specific laminitic damage within the hoof (Garner et al., 1975; Bailey et al., 2003).

The utilization of sugar absorption tests (SAT) in detecting increased gastrointestinal permeability in human clinical diagnosis has been utilized extensively for the past several decades. The utilization of sucrose has been employed for gastric ulceration identification in the horse with limited success (D'Arcy-Moskwa et al., 2012; Hewetson et al., 2006; O'Conner et al., 2004). To date however, entire tract gastrointestinal sugar markers have not been evaluated for use in the horse. Our hypothesis was that high concentrate low forage diets would increase the percent excretion of sugar markers as a result of increased gastrointestinal permeability markers, decrease fecal pH, and packed cell volume and total plasma protein would increase in response to a large concentrate meal in comparison to horses fed a low concentrate high forage diet. Additionally, it was hypothesized that the colostrum supplements would mitigate the hypothesized increase in permeability during the high concentrate low forage diets.

## **Materials and Methods**

#### Horses

The Pennsylvania State University owned the animals utilized throughout the duration of this study and the experiment was approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC #37483). Nine Quarter Horse yearlings, four females and five gelded males, were utilized in a replicated Latin square design 84 d in length. The study consisted of three 28-d periods during the months of October thru December; each period consisted of a 14-d washout interval followed by a 14-d treatment interval. Horses were individually housed at all times and forage and concentrate refusals recorded. Body weight was measured weekly. At the start of the experiment, horses were  $546 \pm 15$  d of age and weighed  $385 \pm 19$  kg (mean  $\pm$  SD). During the initial 16 days of the study rectal body temperatures were taken intermittently, upon the  $18^{\text{th}}$  day of the study forward, body temperatures were taken while horses consumed the morning concentrate meal (between 0730 and 0930 h) and afternoon concentrate meal (between 1630 and 1730 h).

# Treatments

Horses were fed either a low concentrate high forage (LCHF) diet or a high concentrate low forage diet (HCLF) throughout the twelve-week period, alternating every 14 d. Concentrate and hay rations (Table 2-1; Table 2-2) were fed twice daily at 0800 h and 1700 h in equal quantities. During the LCHF washout intervals horses were fed 0.9 kg of the Low Concentrate (LC) and 4.0kg hay per meal (values reported on an as-fed basis). During the HCLF treatment intervals, horses were fed 2.5 kg of the High Concentrate (HC) and 2.0 kg hay per meal (values reported on an as-fed basis). To encourage maximum consumption of concentrate meal, hay was fed to horses following completion of meal or 60-minutes post meal if concentrate refusals remained. Within the HCLF treatment intervals, horses were additionally supplemented with one of three topdressed colostrum treatments; 70 g whole bovine colostrum (WBC), 70 g concentrated bovine colostrum (CBC), or no colostrum (NC). Grain refusals were recorded initially 60 minutes following each concentrate meal to best quantify starch intake following meal. Any remaining refusals were then given back to the horse to allow for further consumption. Final refusals were again recorded prior to feeding the following meal, this allowed for a more appropriate total dry matter intake calculation.

Blood sampling, fecal collection, and sugar absorption tests were conducted throughout the 12-week study. Figure 2-2 provides details of sampling during each interval. On non-sample collection days, horses were individually housed in partially covered paddocks. Horses were fed forages and concentrate twice daily with ad libitum access to water. On blood and fecal sampling days (day 7 of 14 day interval) horses were brought into individual box stalls and fed meals twice daily. Horses were returned to individual paddocks between 1600 and 1800 h. On urine collection days (day 14 of each interval), horses were fasted for 16 hours prior to administration of sugar markers. Horses were brought into individual box stalls at 0530 h and remained in stalls until following day at 0830 h to facilitate total 24-hour urine collection. Figure 2-3 provides a detailed description of urine collection sampling. Horses were not fed any concentrate meals on urine collection days and were fed 2.0 kg hay (as fed value) every four hours beginning two hours after sugar administration; total dry matter intake is reported in Table 2-4. On urine collection days, horses were not allowed access to water from the hours of 0530 to 1000 h. For the remainder of the urine collection horses were allowed ad libitum access to water.

#### Blood sampling

Weekly jugular blood samples were drawn from each horse. Mid-interval (d 7) samples were taken to evaluate the effect of diet on packed cell volume (PCV) and total plasma protein (TPP) parameters. PCV (packed red blood cells as a percentage of whole blood) and TPP (g/dL) were measured prior to the morning grain meal at (0800 h) and 90 minutes after grain meal (0930 h). Blood was drawn into a 12-ml potassium ethylenediaminetetraacetic acid (EDTA) vacutainer tube from the jugular vein, gently inverted 5-10 times, and then stored on ice (BD Vacutainer, Franklin Lakes, NJ). Tubes were periodically inverted prior to PCV measurement to prevent the premature separation of plasma. Within an hour of collection, whole blood was drawn from the recently inverted EDTA vacutainer tubes prior to centrifugation for PCV analysis by microhematocrit centrifugation (13,460 × g for 5 min). Vacutainer tubes were centrifuged at 4°C at 1400 x g in order to separate plasma from the whole blood sample. TPP concentration was then measured using an optical refractometer (ATAGO SPR-T2; Atago Co., Ltd., Tokyo, Japan).

# Sugar Absorption Tests

Total 24-hour urine collections were performed at the end of every 14 d interval. Horses were fasted for 16 hours prior to nasogastric administration of sugar markers. The sugar mixture contained 16.0 g mannitol, 28.0 g sucrose, 28.0 g lactulose, and 80.0 g sucralose dissolved and refrigerated in 500 ml of distilled water 12 to 24 hours prior to administration. These amounts were calculated by body weight dosages reported in the literature and previous pilot work in our lab. Amounts administered were calculated utilizing an average bodyweight of 400kg. Horses were sedated with 1.5 to 2.0 cc romifidine hydrochloride (Sedivet®) based on reactiveness prior to nasogastric tubing of sugar mixture. Horses were fed a total of 12 kg hay throughout each 24-hour period, beginning 2 hours after sugar markers were administered and every four hours following. Average intake per period reported in Table 2-4. Horses were acclimated to urine collection devices prior to the collection periods. Horses wore urine collection devices for the entirety of the 24-hour period following sugar mixture administration to allow for total urine collection. Individual urinations were measured and a 1ml preservative (10% thymol in non-diluted isopropanol) added per 100 ml urine volume. Six hour pooled urine samples (0 to 6 hr, 7 to 12 hr, 13 to 18 hr, and 19 to 24 hr) and a final total 24-hour urine sample were taken. These samples sat at ambient temperature in the barn over the 24hour period. Aliquots were then frozen at -20° Celsius until further analysis.

## Permeability Marker Analysis

Standards of sugar markers were dissolved in triple distilled water, aliquotted into 2.0 ml quantities, and frozen at -20° C. These were then utilized to validate consistent retention times, modify GC-run settings, and quantify standard curves. Details for GC validation are provided within Appendix A.

Urine samples and standards were thawed at room temperature and derivatized via an alditol acetate preparation for analysis via gas chromatography equipped with a flame ionization detector (GC-FID). See Appendix A for a detailed description of this procedure. The detector temperature was 300° C and the injector temperature was 240° C. The initial column temperature of 100° C was held for 2 min and then increased at a rate of 10° C/min to 180° C, which was held for 2 min and then increased at a rate of 10° C/min to 180° C, which was held for 2 min and then increased at a rate of 4° C/min to 240° C, which was maintained for 15 min. The total run time was 42 min. All chromatographic analysis was performed on an Agilent GC-FID 7890. Approximate retention times of each marker and further details on air flow are provided in Appendix A. Retention time of each compound is subject to change with modifications to temperature profile, carrier gas flow rate, column length, and usage of column.

Area underneath the peak of each respective sugar was utilized to calculate the amount of sugar within the 100 microliter urine sample derived from a standard curve (Appendix A). This sugar weight was then adjusted to accurately reflect total urine volume from that specific sample, providing total sugar excreted. Total sugar excreted was then divided by the weight of sugar administered at the start of the test in order to provide the percent excretion of oral dose.

## Statistical Analysis

All data were analyzed via a mixed model ANOVA utilizing a PROC MIXED procedure with an autoregressive covariate structure and Tukey's post-hoc test to determine differences of statistical significance within SAS version 9.2 (SAS Institute Inc., Cary, NC). Severe outliers were removed from each data set utilizing STATA version 10.1. Severe outliers defined as those values less than or greater than three times the inner quartile range. For all statistical analyses  $P \le 0.05$  was considered statistically significant and  $P \le 0.10$  was considered a statistical trend. Nutrient composition data are reported as means  $\pm$  standard deviation. All other variables reported as means  $\pm$  standard error, unless specified otherwise. Time variables have been identified previously and are illustrated in Figures 2-1, 2-2, and 2-3. Two main comparisons made, a supplemental and a dietary comparison. The supplemental comparison refers to differences of WBC, CBC, and NC within the treatment HCLF intervals only. The dietary comparison refers to differences between the two main dietary differences of LCHF to HCLF. For this main dietary comparison, supplement groups during the HCLF diet were pooled.

## Dietary Intake

All dietary intake parameters (1-hour post meal and total DM grain and hay intakes) were evaluated utilizing the following analyses. The effects of diet, period, and diet by period interaction were evaluated by a mixed model ANOVA with fixed effects of diet, period, and the diet by period interaction with a random effect of horse. The final two days of each interval were removed from dietary intake analyses due to the alterations in feeding patterns for the completion of sugar absorption tests. Additionally, total dry matter intake was calculated for urine collection days separately.

## Blood parameters

To evaluate the change in packed cell volume and total plasma protein based on response to diet, pre-meal and 90 min post-meal samples were taken. To calculate difference the pre-meal value was subtracted from the post-meal value, this allowed for the elimination of individual variation. A mixed model ANOVA evaluated the effects of diet, period, and the diet by period interaction. Fixed effects of diet, period, and diet by period interaction were utilized. The random effect included horse within period and interval was utilized for the repeated statement.

## *Body temperature*

In order to eliminate variation in basal body temperature across the nine horses, body temperature was evaluated by difference. An average body temperature was calculated for the each 14 d LCHF washout interval. This average was utilized to correct for individual variation and was subtracted from the actual recorded rectal temperature during both the LCHF and HCLF intervals. The change from LCHF average was calculated within each period. The reported values are the degree change from average washout interval temperature. The effects of diet and period were evaluated by a mixed model ANOVA with fixed effects of diet and period. The repeated statement was temperature time within sample day, with a random effect of horse within period. It should be noted that period 1 was removed from analysis due to a lack of data.

## Fecal pH

The effects of diet, sample day within diet, and period were evaluated by a mixed model ANOVA with fixed effects diet, sample day within diet and period. The repeated statement included sample day within period, with a random effect of horse within period.

#### Sugar marker percent excretion

The effect of diet or supplement, period, and the interaction of diet or supplement and period on all pools of urinary excretion were evaluated by a mixed model ANOVA with fixed effects of diet, period, and diet by period interaction. A random effect of horse was additionally utilized. The effect of diet or supplement, period, and the interaction of diet or supplement and period were evaluated utilizing a mixed model ANOVA with fixed effects of diet or supplement, period, and diet or supplement by period interaction. No repeated measures were included within these analyses. Values below or above the validated range of linearity for detection were also removed from analyses. The standard curve for each sugar marker is presented in Appendix A.

# Results

# Intake and body weight

Body weight increased by period (P < 0.0001). Average body weight during period 1 was 395.3 ± 5.7, during period 2 it was 402.7 ± 5.7 kg, and during period 3 horses weighed 407.1 ± 5.7 kg. Body weight by interval is presented in Table 2-6.

Concentrate intake was not different across various supplementation groups (P > 0.10). Dry matter intake of concentrate 60 minutes post-meal for the LCHF intervals was  $0.8 \pm 0.05$  kg. In contrast horses on the HCLF intervals consumed  $1.8 \pm 0.05$  kg (P < 0.0001). Starch intakes for the 60-minute post-meal measurements include  $0.1 \pm 0.02$  kg starch for the LCHF concentrate and  $0.6 \pm 0.02$  kg starch for the HC concentrate, differences of diet (P < 0.0001) and period (P = 0.0012) were significant. Starch intake by period is presented in Table 2-4. During the HCLF treatment intervals, yearlings consumed 1.1 kg more starch per day than during LCHF washout intervals (P < 0.0001). Based on the averages of diet, the yearlings consumed 0.3 g/kg BW of starch per meal on the LCHF concentrate and animals consumed 1.6 g/kg BW of starch per meal on the HCLF concentrate. Summarized dry matter intake of starch intakes from concentrate are reported in Table 2-4.

Forage intake was not different across various supplementation groups (P > 0.10). Dry matter intake of forage on LCHF per day was 7.0 ± 0.05 kg and on HCLF was 3.5 ± 0.05 kg, differences of diet (P < 0.0001). Forage intake was lower during period 1 (P < 0.0001), values provided in Table 2-3. Total starch intake from forage on LCHF per day was 0.06 ± 0.0004 kg and on HCLF was 0.03 ± 0.0004 kg (P < 0.0001). On the LCHF diet, the combined daily dry matter intake of both concentrate and forage was 2.1% bodyweight. On the HCLF diet, the combined daily dry matter intake of both concentrate and forage was 1.9% bodyweight.

Dry matter intake of forage during 24-hour sugar absorption tests are reported in Table 2-6, differences in interval (P < 0.0001) were significant. The range of intake during the 24-hour period was  $8.6 \pm 0.2$  to  $10.9 \pm 0.2$ , on intervals 6 and 3 respectively. Differences in least square means reveal forage intake during interval 6 was different from all other intervals (P < 0.0001) and interval 3 was different from interval 4 (P = 0.0275).

# Packed Cell Volume and Total Plasma Protein

Packed cell volume decreased 90-minutes following concentrate meal  $0.1 \pm 0.27$ and  $1.0 \pm 0.27$  percent from pre-meal measures on the LCHF and HCLF diets (P = 0.0381), respectively. Total plasma protein at 90 minutes post-meal decreased from premeal measurement  $0.21 \pm 0.05$  g/dL on the LCHF and  $0.18 \pm 0.05$  g/dL HCLF diets (P > 0.10).

## *Body Temperature*

The change in body temperature from washout average during LCHF intervals was  $0.017 \pm 0.030$  °F and change from washout average during HCLF intervals was  $0.304 \pm 0.030$  °F (P < 0.0001). Change during period 2 was less than period 3 (P = 0.0151). Period 1 was not included in analysis.

## Fecal pH

Fecal pH during the LCHF intervals was  $6.9 \pm 0.04$  and higher than during the HCLF diet when it was  $6.5 \pm 0.04$  (P < 0.0001). Fecal pH decreased from day 7 of interval to day 14 of interval (P < 0.0001) regardless of diet. Fecal pH of period 1 was

different from period 2 only as determined by differences of least square means (P = 0.0217). Figure 2-4 represents the dietary comparison of fecal pH.

## Permeability

Percent excretion of the four sugar markers administered; sucrose, lactulose, mannitol, and sucralose, were evaluated based upon supplemental and dietary effect. Additionally, the ratios of both lactulose: mannitol and sucralose: mannitol were evaluated. Each urine collection was represented by a total 24-hour pool, 0 to 6 hour pool, 7 to 12 hour pool, 13 to 18 hour pool, and 19 to 24 hour pool were individually evaluated and are presented as such below. Tables 2-7 through 2-11 provide percent excretion values for all sugars and ratios for supplemental comparisons. Tables 2-12 through 2-16 provide percent excretion values for all sugars and ratios for dietary comparisons. Figures 2-5 through 2-8 provide visual representations by sugar of urine collection pools, with supplemental comparisons made within urine collection. Figures 2-9 through 2-12 provide visual representations by sugar of urine dietary comparisons made within urine collection pools, with dietary comparisons made within urine collection.

#### Percent excretion within 24-hour urine pools

Supplement treatment did not impact the percent excretion of sucrose, lactulose, mannitol, sucralose, or lactulose: mannitol during the complete 24-hour collection (P > 0.10). Whole bovine colostrum, CBC, and NC had sucralose: mannitol of  $0.7 \pm 0.08$ ,  $0.82 \pm 0.08$ , and  $0.94 \pm 0.08$ , respectively (P = 0.0915) (Table 2-7).

Percent excretion of sucrose (P < 0.0001), lactulose (P = 0.0444), and mannitol (P = 0.0009) were different between LCHF and HCLF fed yearlings, values provided in Table 2-12. The sucralose: mannitol as it decreased from  $1.0 \pm 0.06$  on the LCHF diet to  $0.8 \pm 0.06$  on the HCLF diet (P = 0.0524). Percent excretion of sucrose was different by period (P = 0.0192). Specific values provided in Table 2-12. Differences in least squares means did not detect a difference in the percent excretion of sucrose when horses were fed either LCHF and HCLF during period 1 (P = 0.1068), but was significant for periods 2 and 3. Period averages reported in Table 2-12.

## Percent excretion within 0 to 6 hour urine pools

Percent excretion of sucrose during the 0 to 6 hour collection was highest for WBC at  $1.1 \pm 0.10$ , while CBC  $0.4 \pm 0.1$  and NC  $0.7 \pm 0.2$  (P = 0.0543) represented in Figure 2-5 and Table 2-8. Differences of least square means indicate only WBC and CBC percent excretion of sucrose were different (P = 0.0432), however neither was different from NC (P > 0.10). Additionally, percent excretion of sucrose during intervals 2, 4, and 6 were different (P = 0.0425). No other significant supplemental comparisons exist for the remaining sugar markers or ratios at the 0 to 6 hour time point. Although not significant, percent excretion of all sugars during supplementation with WBC is numerically higher than other supplement groups during the 0 to 6 hour collection (Table 2-8).

Percent excretion of sucrose during the 0 to 6 hour urine collection was influenced by diet (P = 0.0197), Figure 2-9. Horses on the LCHF diet had a percent excretion of sucrose of  $0.4 \pm 0.11$  and those on the HCLF diet had a percent excretion of sucrose of  $0.7 \pm 0.08$  (Table 2-13). No other sugar markers or ratios were significantly influenced by diet (Table 2-13).

## Percent excretion within 7 to 12 hour urine pools

Percent of mannitol trended towards significance when supplemental groups were compared (P = 0.0732), as shown in Table 2-9 and Figure 2-7. Yearlings supplemented with whole bovine colostrum had a percent excretion of oral dose of mannitol of 0.7 ±

0.08, while yearlings supplemented with CBC or NC had percent excretion of oral dose of mannitol values of  $0.4 \pm 0.09$  and  $0.6 \pm 0.08$ , respectively. Differences of least squares means revealed that the only supplemental comparison for the percent excretion of oral dose of mannitol that trended towards significance (P = 0.0635) was that of WBC and CBC. Percent excretion of oral dose of sucralose was different for supplement (P =0.0457); highest during supplementation with WBC ( $0.5 \pm 0.04$ ), followed by NC ( $0.4 \pm$ 0.04), and lowest in animals supplemented with CBC ( $0.3 \pm 0.04$ ) as shown in Figure 2-8. As was observed in the trend in mannitol previously discussed, differences of least squares means identified a specific significance between WBC and CBC for the percent oral dose excretion of sucralose (P = 0.0395) during the 7 to 12 hour collection. No other differences in sugar markers by supplemental comparisons were observed (Table 2-9).

No dietary differences were observed in percent excretion of individual sugar markers or ratios during the 7 to 12 hour time point (Table 2-14).

## Percent excretion within 13 to 18 hour urine pools

No significant differences were observed for individual sugar percent excretions of oral dose or ratios were observed for supplemental comparisons during the 13 to 18 hour urine collection. Interestingly, the measure comparison of sucralose: mannitol on HCLF diet was statistically significant (P = 0.0095). These comparisons are displayed in Table 2-10.

During the 13 to 18 hour urine collection, there was a trending increase when yearlings were fed the HCLF diet in percent excretion of both lactulose (P = 0.063) and mannitol (P = 0.0769). Additionally, the sucralose: mannitol of percent excretion during the 13 to 18 hour urine collection in yearlings fed the HCLF diet trended in significance (P = 0.0641) (Table 2-15).

Percent excretion within 19 to 24 hour urine pools

No differences in percent excretion of individual sugar or sucralose: mannitol by supplement group were observed during the 19 to 24 hour urine collection (Table 2-11). The lactulose: mannitol was lowest for yearlings supplemented with WBC and equal for CBC and NC (P = 0.0077) (Table 2-11). A trend in significance was observed within intervals of the HCLF diet for sucralose (P = 0.0915), no other significant differences were observed.

Yearlings fed the HCLF diet had higher percent excretion of lactulose than those fed the LCHF diet (P = 0.0535). Percent excretion of lactulose was  $0.3 \pm 0.04$  on LCHF diet and  $0.4 \pm 0.04$  during HCLF diet for the 19 to 24 hour urine collection. Additionally, differences in period for percent excretions of sucrose (P = 0.0262), lactulose (P = 0.0496), and sucralose (P = 0.0191) were observed. Values presented in Table 2-16.

# Discussion

Our hypothesis was that an increase in gastrointestinal permeability on high concentrate low forage diets could be mitigated with supplementation of bovine colostrum. The exact mechanism via which bovine colostrum may serve to heal and protect the gastrointestinal barrier is largely unresolved, however the roles of several biological factors have been indicated as cellular growth promoters. Nonpeptide trophic factors, hormones, cytokines, and growth factors are all components of colostrum thought to impact gastrointestinal growth (Playford et al., 2000). An additional hypothesis was that the HCLF diet would increase gastrointestinal permeability and decrease fecal pH in comparison to horses fed the LCHF diet.

The negative impact of high starch, low forage diets on gastrointestinal permeability is often hypothesized in equine models, but had yet to have been measured

in a whole gut model in the horse in vivo. Two major concerns within the equine industry regarding the consequences of feed management include ulceration (O'Conner et al., 2004) and feeding of large quantities of fermentable carbohydrates (Al Jassim and Andrews, 2009), such as excess of starch by concentrate. The broader applicability of this objective lies in the usefulness of the SAT in a variety of equine research models in order to identify subtle changes that may occur in various disease states or athletic models sub-clinically prior to physical presentation. The findings from this work provide evidence that this technique has the potential to provide new diagnostic measures of equine gastrointestinal health.

The initial objective of this work was to evaluate the effectiveness of dietary supplementation with two forms of bovine colostrum hypothesized to reduce gastrointestinal permeability induced by a high starch, low forage diet, in comparison to a non-supplemented control group. Bovine colostrum has been effectively utilized in both exercise performance models and human clinical treatments for NSAID induced ulceration and other gastrointestinal disease states (Playford et al., 1999; Uruakpa et al., 2002; Shing et al., 2009). This study provides evidence that the twice-daily supplementation of 70.0 g of either WBC or CBC did not significantly influence gut health as hypothesized, however the data indicates that CBC may be worth further investigation as an equine gut health supplement. The twice-daily dose of colostrum was selected based on pilot work conducted prior to this study. More than 70.0 g of CBC or WBC top-dressed over the concentrate would have led to a decline in palatability.

The two diets fed during this project allowed for major differences in both starch and forage intake, however the possibility of other dietary components impacting results observed cannot be eliminated. A major factor that may have impacted the digestibilities of both concentrate and forage was the inclusion of the yeast product Levucell SC, a Saccharomyces cerevisiae feed additive. This product has been shown to improve digestibility of acid detergent fiber and neutral detergent fiber in horses supplemented with 10 g/d (Jouany et al., 2008). Levucell SC concentrations were not quantified in either concentrate preparation and should be avoided in future studies attempting to exceed the capacity of the small intestine. Limit feeding of forages has been shown to increase gastric ulceration (Murray and Eichorn, 1996; O'Conner et al., 2004). This is likely due in part to the continuous acid secretory mechanisms that exist within the equine stomach (Campbell-Thompson and Merritt, 1990) and the lack of salivary bicarbonate and feed contents within the gastric lumen. While the horses did not undergo endoscopy at any point in time over the 12 week period, the dietary difference observed in the oral percent excretion of orally dosed amounts were significant at both the 24-hour total period and the 0 to 6 hour period providing evidence that gastric ulceration may have been increased on the HCLF diets. The utilization of sucrose as a marker of gastric permeability has been implemented with some success in the horse previously (O'Conner et al., 2004; Hewetson et al., 2006; D'Arcy-Moskwa et al., 2012). Urinary excretion of sucrose was accurately utilized to detect ulcer scores greater than 1. A score of zero was indicative of intact mucosal epithelium and a score of three indicated ulceration through the mucosal layer (O'Conner et al., 2004). Bladders were emptied via urinary catheter to allow for a baseline urine sample to be collected. Horses were then fed 1 kg of a 10% protein pellet (Horsechow 100; Purina Mills) just before they received 454 g of sucrose (10% solution in tap water) via nasogastric tube. Urine was then collected at 2 and 4

hours post administration of sugar via urinary catheter. Sucrose concentration in urine was reported in a range of 0.06 to 5.58 mg/mL as detected utilizing high performance liquid chromatography with pulsed amperometric detection (O'Conner et al., 2004). It follows that due to differences in protocol our findings are lower than reported previously in urine. With improvements in detection technology, detection of sucrose in serum has additionally been investigated (Hewetson et al., 2006; D'Arcy-Moskwa et al., 2012).

It has been shown previously that size, rate of consumption, and concentrate meal frequency have the potential to influence fluid balance within the horse, although the mechanisms under which fluid balance are regulated are quite complex (Youket et al., 1985; Clarke et al., 1988; Houpt et al., 1988). Horses (384-516 kg BW) fed a large single concentrate meal with an average consumption of 4.0 kg at 63% of the amount fed, in contrast to those fed six, equally distributed meals totaling 6.3 kg possessed different biological responses to meal size and frequency. These diets consisted of a 12.5% pelleted crude protein with no mention of starch content or forage availability throughout these periods, likely as the PCV and TPP measurements were part of a larger objective investigating the renin-angiotensin system in the horse. TPP and PCV were evaluated in response to both feeding strategies. PCV and TPP in horses fed six small, approximately 1.0 kg meals did not change over time in response to meal. Contrastingly, PCV and TPP in animals fed the single large meal, approximately 4.0 kg, were different (P < 0.05) from pre-feeding values at 5 and 7 hours post meal and 30 and 60 minutes post meal, respectively (Clarke et al., 1988). Packed cell volume change from baseline was greatest 5 hours after the meal and increased from  $29.1 \pm 1.0$  to  $36.3 \pm 3.0$  percent. Interestingly, the decrease in plasma volume did not correspond with the initial increase in TPP

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observed, which increased from 7.6  $\pm$  0.4 g/dL pre-feeding to 8.5 g/dL at 30 minutes post-feeding (Clarke et al., 1988). The later change in PCV was associated with colonic secretions required for digestion. Similar changes in TPP and PCV were observed in a similarly designed study (Houpt et al., 1988). Shetland ponies fed one single large meal at 1.2% body weight exhibited an increase in TPP, 8.1 g/dL, during the hour in which the animals were eating, followed by a decrease until 90 minutes post-meal when values returned to those similar to pre-feeding, 7.1 g/dL (Houpt et al., 1988). 30 minutes into the meal, PCV increased from approximately 33 to 37 percent (P < 0.001). This decrease in plasma volume, and thus increased concentration of plasma protein following a concentrate meal has been previously attributed to increased alimentary secretions and osmolality shifts based on increases in acidity within the hindgut. This is supported by the Houpt et al. (1988) data time course.

Another consequence of large meal feeding of concentrates is the disturbance in luminal pH that results from fermentation of various substrates by the microbial population within the hindgut. Lopes et al., (2004) investigated the impact of large grain meals (4.55 kg at 30.6% starch) on various cecal and fecal parameters within the horse. Horses were either fed ad libitum hay, adapted to hay prior to the addition of 4.55 kg high starch grain with ad libitum forage, or adapted to both hay and grain and fed the 4.55 kg high starch grain with ad libitum forage. Rectal body temperature during this study was different by diet, however the biological significance of this study should be questioned much like our results, as the reported difference is less than a half of a degree Celsius (Lopes et al., 2004). In our study, the body temperatures of each horse on the HCLF diet was on average 0.3°F higher than the average measure during the LCHF diet. These differences should be interpreted carefully however; as statistical significance may be influenced by the frequency of measurement and does not always indicate biological significance. Conversely, body temperature is very tightly regulated within any mammal and this slight increase may be indicative of subclinical events occurring during the HCLF diet that are not present on the LCHF diet.

In 1990, Clarke et al., hypothesized that the episodic meal feeding and associated decrease in lumen pH currently implemented in equine husbandry may lead to damage of the epithelial barrier and initiate an immune response. The decrease in fecal pH during the HCLF periods of our study indicates that the fermentative products were different on the two dietary treatments. The average fecal pH of the LCHF diet was  $6.9 \pm 0.0$ , in comparison to horses fed the HCLF diet, which was  $6.5 \pm 0.0$ . The minimum pH recorded within our animals was 6.04 and the maximum 7.47. These values are similar to those observed within Australian racehorses with a mean of 6.5, minimum of 5.5 and maximum of 7.9 (Richards et al., 2006).

While a variety of strong supporting evidence from changes in microbial populations (Milinovich et al., 2007; Respondek et al., 2008), the appearance of plasma endotoxin (Sprouse et al., 1987), and in vitro measurement of permeability (Kreuger et al., 1986; Weiss et al., 2000), have been examined to support this carbohydrate induced leaky gut theory, direct measures of gastrointestinal permeability have not yet been evaluated in vivo in the horse. The SAT has been used extensively in human disease identification and research models, ranging from work on irritable bowel syndrome to exercise stress models in soldiers (Bjarnason et al., 1995; Li et al., 2013). While the premise of SAT may seem simple, there are a variety of factors that influence the rate and

extent of absorption, even in the human field where SAT have been extensively used there is still debate as to the most appropriate timing of sample collection (McOmber et al., 2010; Rao et al., 2011). Markers have been identified within the colon of human subjects as early as 2 hours post administration (Rao et al., 2011). Due to the preliminary stages of this technique in the horse the importance of extensive data collection is critical to the interpretation of these markers.

The sugar absorption test has not been utilized previously in the horse. The amounts of sugar markers administered during this project were kept constant over the 12-week study period, but were developed on a bodyweight dose originally. Differences in percent excretion of sucrose were observed by diet in total 24-hour and 0 to 6 hour samples. 90% of excreted sucrose was recovered by 6 hours in children and adults (McOmber et al., 2010). A similar pattern of excretion in the horse was observed within the 0 to 6 hour urine pool. Unexpectedly, there was a consistent increase in sucrose excretion from the 0 to 6 hour samples to the later 6-hour pooled urine samples. Unfortunately labeled sucrose was not utilized and thus no definite conclusion of sucrose source can be determined, however an explanation of the increase observed at the later time points may be a result of hay digestion or perhaps slower renal clearance into the urine. We do not know the source or growth conditions of our mature mixed grass hay, however sucrose content as measured in Timothy hay from various cuttings and locations was reported to range from 15.0 to 58.7 mg of sucrose per g DM. It should be acknowledged that the NSC content reported in this work is several fold higher than what was fed on this study (Pelletier et al., 2009). The prior explanation is more likely

however as the 0 to 6 hour pool fits previous sucrose excretion studies in the horse and human excretion profiles (O'Conner et al., 2004; McOmber et al., 2010).

Lactulose, another disaccharide, is a representative marker of gastric and small intestinal damage. Human sugar absorption tests have previously found that by 16 to 18 hours post sugar administration, 90% of the lactulose excreted over a 24-hour period had been excreted in the urine (McOmber et al., 2010). Contrastingly, lactulose has been shown to appear in the human colon within 2 hours of administration, further complicating the interpretation of these sugar tests (Camilleri et al., 2010). The results from this study indicate that over the 24-hour total collection lactulose excretion was different between the HCLF and LCHF diets, specifically during the 13 to 18 hour and 19 to 24 hour measurements. This brings in to question the length of time required to ferment lactulose. Fluid markers were cleared from the small intestine by 12 hours in ponies (Argenzio et al., 1974). Given the excretion rates of lactulose remained fairly consistent between the hours of 7 and 24 of the 24-hour collection it is difficult to identify if these excretions were from the small or large intestine. Percent excretion was similar between diets during the first 12 hours of excretion and was greater than the last 12 hours of excretion where a dietary difference was observed. Percent excretion of lactulose over the full 24-hour period was 2-fold higher within animals on the HCLF diet. It is unlikely that this difference is due to gastric permeability alone because there was no difference in the 0 to 6 hour samples. This supports the theory that small intestinal damage may have occurred within the horses on the HCLF diet. Due to differences in rate of passage from length and digestive function of the equine gastrointestinal tract in comparison to the human it is likely that a longer period of time is reflective of small

intestinal transit (Argenzio et al., 1974). No supplemental differences were observed under the evaluation of percent excretion of lactulose.

The only monosaccharide administered in most SAT, mannitol, is utilized as a correction factor for surface area as it, unlike disaccharides, has the capability of passing through cells rather than solely through the junctions in between cells, although this too has been debated (Bjarnason et al., 1995). The findings of this study indicate that mannitol excretion was different by diet over the total 24-hour period and additionally at the 13 to 18 hour time point. This may indicate differences in apparent surface area or mucosal integrity. The influence of high starch diets on villus height within both the rumen and small intestine of ruminants has been previously documented, although the presence of hydrolysable carbohydrates can have both a positive and negative impact on epithelial condition (Wang et al., 2009; Steele et al., 2011). The impact of three levels of dietary starch inclusion upon rumen and small intestinal morphology were investigated in goats (Wang et al., 2009). Dietary starch was included at 28, 35, and 46%; these inclusions caused ruminal pH to decline to 6.34, 6.30, and 6.00, respectively (P = 0.027). Rumen morphology was evaluated in both the ventral ruminal sac and the dorsal ruminal sac via the following measurements: papillae height, width and surface, as well as wall thickness and papillae density. The 35% starch diet increased papillae height in the ventral rumen sac (P < 0.001), while 46% starch diet decreased papillae height in the dorsal rumen (P = 0.001). The 35% starch diet additionally increased papillae surface, while the 46% starch diet resulted in the smallest papillae surface (P < 0.001). No statistical differences in pH were observed at any portion of the small intestine. Villus height and width, crypt depth, and villus height to crypt ratio, and villus surface area were each evaluated. No differences were observed in any period except for an increase in villus height in the jejunum while fed the 35% starch diet (Wang et al., 2009). In several subacute ruminal acidosis models in dairy cows a decrease in pH may damage ruminal epithelium and increase permeability to LPS (Gozho et al., 2005; Steele et al., 2011; Plazier et al., 2012). Acidotic (pH 5.1) in vitro environments induced increased ovine rumen permeability (Aschenback and Gäbel, 2000). Due to the stark differences in amount and type of substrate available on the two diets in the present study, it is possible that they influenced the surface area within the gastrointestinal tract of the horses. This would support the differences observed in mannitol excretion over the total 24-hour pool and at the 13 to 18 hour time point as this could represent distal small intestine or hindgut measures.

It is common to utilize the ratio of disaccharide to monosaccharide in the evaluation of gastrointestinal permeability, and the most common comparison is that of lactulose: mannitol (Dastych et al., 2008). The interpretation of this ratio is perhaps one of the most debated recent topics in utilizing sugar absorption tests as it can be influenced by many factors and thus does not provide a clear answer as to the extent of damage (Camilleri et al., 2010; Rao et al., 2011). No differences within the dietary comparisons of lactulose: mannitol excretion were observed in our data, however the ratio of lactulose to mannitol was different across supplemental groups at the 19 to 24 hour portion of the sugar absorption test. The ratio was lower when animals were supplemented with whole bovine colostrum in comparison to the more equal ratios observed on CBC and NC treated horses. When compared to the percent excretions of individual sugars mannitol and lactulose, each of the supplement groups follows the same trend, however the percent

excretion values for individual sugars are lower on the whole bovine colostrum supplement. It is possible that there were general differences in surface area between the whole bovine colostrum supplement and the others resulting in differences in percent excretion of mannitol. When individual comparisons of supplemental groups are examined, the lactulose: mannitol under whole bovine colostrum supplementation was lower than the ratios observed for CBC and the NC groups (P < 0.02). The CBC group and NC group however were not different from one another (P > 0.10). These ratios are often difficult to interpret and based upon the lack of difference in the individual sugars should not be of primary focus of conclusions. The differences observed here are likely due to non-statistically difference fluxes in mannitol excretion.

Finally, the disaccharide that is indicative of whole gut permeability is sucralose. Sucralose is not degraded by bacteria or enzymatic degradation (Farhadi et al., 2003). In comparison to the previously mentioned markers, sucralose is relatively recent in regard to evaluating gastrointestinal permeability. Additionally, much of the work in human clinical medicine has focused on small intestinal damage and the utilization of the lactulose: mannitol. The percent excretion of sucralose was not influenced by diet (P >0.10). This is possibly due to a lack of damage caused within the hindgut, the yearlings were not fed starch over the currently proposed levels necessary for starch overload and is supported in supplemental findings discussed below (Potter et al., 1992; Kienzle, 1994).

Supplemental comparisons of the percent excretion of sucralose were different at the 7 to 12 hour period of the sugar absorption test. The highest percent excretion within animals administered the WBC and lowest with animals supplemented with CBC. This is interestingly the same trend observed for sucrose excretion during the 0-6 hour period. The only other trend in significance of sucralose percent excretion remained in the 19 to 24 hour urine collection, however it was by interval on the HCLF diets. The HCLF diet during the second period resulted in the highest percent excretion of sucralose, while the HCLF diet within the third period was the lowest. Contrastingly there were no differences in percent excretion of sucralose across washout LCHF intervals. This suggests that perhaps inconsistent damage to the hindgut occurred on the HCLF diets. This conclusion is not unlikely, as the yearlings were not consuming starch at levels currently proposed to exceed starch digestion within the small intestine. The yearlings on this study consumed an average of 1.6 g/kg BW of starch on a body weight basis while 2.0 and 3.5 g/kg BW of starch on a body weight basis while 2.0 and 3.5 g/kg BW of starch on a body weight basis while 2.0 and 3.5 g/kg BW of starch on a body weight basis while 2.0 and 3.5 g/kg BW

The most recent permeability ratio to be included in the literature is that of the sucralose: mannitol ratio as suggested by Farhadi et al., in 2003. Dietary comparison of the sucralose: mannitol ratio revealed a trend in significance for the complete 24-hour urine pool, 13 to 18 hour urine pool, and significant difference at the 19 to 24 urine pool. The interpretation of significance of this ratio however, should be utilized with caution much like that of the lactulose to mannitol ratio (Camilleri et al, 2010). There were no dietary differences observed during any of the above-mentioned collections for sucralose percent excretion, differences were observed however for mannitol percent excretion. This again questions the appropriateness of drawing conclusions from a ratio involving mannitol as a correction factor (Camilleri et al., 2010).

# Conclusion

Supplementation with WBC did not attenuate increases in permeability as induced by HCLF diets, yet the results support further investigation of CBC. As evidenced by the increases in percent excretion of sucrose, lactulose, and mannitol over the 24-hour pool yearlings fed the HCLF diet had higher gastrointestinal permeability than those fed the LCHF diets. The lack of difference between diets for sucralose indicates that perhaps the level of starch fed was not sufficient to cause hindgut gastrointestinal damage. Alterations in gastrointestinal permeability have the potential to elicit a variety of gastrointestinal disease states and immune response. In order to further understand the impact of equine nutritional management upon overall health of the horse we need to further investigate the utilization of non-invasive techniques. The SAT provides one mode of investigation, while pursuit of changes in microbial communities, metabolomics, and inflammatory markers are additionally being investigated. Admittedly, the major limitation to the SAT is the interpretation of marker excretion. In its current stages, this test provides us with a preliminary guestimate of site damage within the gastrointestinal tract of the horse and has allowed for comparative analysis of dietary treatments.

These preliminary results indicate that the sugar absorption test has the potential to identify subtle changes in gastrointestinal permeability within the horse. Extensive validation will be required before it is applicable outside of a controlled research setting, however the validation of this tool should be pursued. Particular components including separation of sucrose marker from dietary sucrose, time course of marker passage, evaluation of age, sex, and breed differences, and fermentability of markers within equine digesta would provide more information with regard to moving forward. Additionally, it may be beneficial to utilize a simpler study design under which known damage is

occurring throughout specific locations of the gastrointestinal tract for comparison to percent excretion values.

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Ingredient, % <sup>2</sup>	LC	HC
Custom mixer pellet <sup>3</sup>		66.2
Cracked corn		17.9
Molasses		7.0
Beet pulp		5.0
Oats		3.8
ToxGaurd		0.1
Tribute Performance Advantage <sup>4</sup>	55.6	
Kalmbach 32% Supplement Pellet <sup>4</sup>	44.4	

**Table 2-1.** Ingredient composition of concentrates fed to long yearling Quarter Horses<sup>1</sup>.

 <sup>1</sup> Ingredient information was provided by Kalmbach Feeds, Inc.
 <sup>2</sup> Composition of concentrates was constant throughout the 12-week study.
 <sup>3</sup> 65.1% of pellet consists of fine ground corn, corn starch, and corn distillers dried grains. This pellet also contained a yeast product called Levucell SC®. Details provided in Appendix B.

<sup>4</sup>Available from Kalmbach Feeds, Inc.

	Conce	entrates	
Item	LC	HC	Mixed Grass Hay
DM, %	$90.9 \pm 0.4$	$90.3 \pm 0.3$	$93.4 \pm 0.2$
CP, %	$25.5 \pm 0.5$	$16.9 \pm 1.2$	$6.7 \pm 0.2$
$ADF^2$ , %	$10.5 \pm 1.1$	$5.7 \pm 0.2$	$38.7 \pm 1.0$
$NDF^3, \%$	$16.2 \pm 0.7$	$10.8\pm0.9$	$59.4 \pm 3.3$
Starch <sup>4</sup> , %	$15.9 \pm 1.5$	$32.0 \pm 1.0$	$0.8 \pm 0.5$
$WSC^5$ , %	$13.9 \pm 0.7$	$14.2 \pm 1.2$	$9.3 \pm 0.5$
$NSC_{-}^{6}, \%$	$29.8 \pm 1.1$	$46.1 \pm 0.6$	$10.1 \pm 0.4$
$\mathrm{ESC}^7,\%$	$8.9 \pm 1.9$	$7.0 \pm 1.3$	$5.8 \pm 0.8$
Crude Fat <sup>8</sup> , %	$7.7 \pm 0.3$	$4.3 \pm 2.0$	N/A
NFC <sup>9</sup> , %	$40.6 \pm 1.0$	$62.7 \pm 4.1$	$22.7 \pm 3.12$
Ash, %	$11.6 \pm 0.1$	$6.5 \pm 0.3$	N/A
Ca, %	$2.1 \pm 0.1$	$1.0 \pm 0.1$	$0.6 \pm 0.0$
P, %	$1.2 \pm 0.1$	$0.7 \pm 0.0$	$0.2 \pm 0.0$
Mg, %	$0.5 \pm 0.3$	$0.2 \pm 0.0$	$0.3 \pm 0.0$
K, %	$1.4 \pm 0.4$	$1.1 \pm 0.1$	$1.2 \pm 0.1$
Na, %	$0.3 \pm 0.0$	$10.4 \pm 17.7$	$0.03 \pm 0.0$
Fe, ppm	$750.6 \pm 21.6$	$465.7 \pm 24.0$	$70.7 \pm 6.4$
Zn, ppm	$309.3 \pm 14.2$	$302.0 \pm 27.1$	$17.7 \pm 2.1$
Cu, ppm	$127.1 \pm 3.3$	$99.3 \pm 7.4$	$6.0 \pm 1.0$
Mn, ppm	$196.2 \pm 12.4$	$167.3 \pm 17.2$	$81.0 \pm 16.1$
DE <sup>10</sup> , Mcal/kg	$4.1 \pm 0.0003$	$3.6 \pm 0.0353$	$2.5 \pm 0.0495$

**Table 2-2.** Nutrient composition<sup>1</sup> of concentrates and hay.

Presented as means  $\pm$  standard deviation, on a dry matter basis. For all presented values, n = 3.

<sup>1</sup> DM basis, as analyzed by Dairy One Forage Lab (Ithaca, NY).

<sup>2</sup> Acid detergent fiber: cellulose and lignin component.

<sup>3</sup> Neutral detergent fiber: hemicelluloses, cellulose, and lignin component.

<sup>4</sup> Starch: polysaccharide component.

<sup>5</sup> Water soluble carbohydrate: monosaccharides, disaccharides, and some polysaccharides (fructans).

<sup>6</sup> Non-structural carbohydrate: starch + water soluble carbohydrate.

<sup>7</sup> Ethanol soluble carbohydrate: monosaccharides and disaccharides extracted in 80% ethanol.

<sup>8</sup> Crude fat: determined by ether extraction.

<sup>9</sup> Non-fibrous carbohydrate: an estimate of non cell wall carbohydrate.

NFC = 100 - (CP% + NDF% + Crude Fat % + Ash %).

<sup>10</sup> Digestible energy, as calculated by the 2007 NRC:

DE = (2,118 + 12.18(%CP) - 9.37(%ADF) - 3.83(%ADF-%NDF) + 47.18(Crude fat %) + 20.35(100 - %NDF - %Crude fat - %Ash - %CP) - 26.3(%Ash))/1000.

			Conce	ntrate			For	age			Total Dr	y Matte	Я
			LC		HC		LC		HC		LC		HC
Period	Average BW	kg/d	%BW/d	kg∕ d	%BW/d	kg∕ d	%BW/d	kg/ d	%BW/d	kg∕ d	%BW/d	kg/ d	%BW/d
1	395	1.42	0.36	3.99	1.01	6.83	1.73	3.52	0.89	8.25	2.09	7.51	1.90
2	403	1.64	0.41	4.02	1.00	7.11	1.76	3.65	0.91	8.75	2.17	7.67	1.90
3	407	1.64	0.40	3.90	0.96	7.16	1.76	3.62	0.89	8.80	2.16	7.52	1.85
Average dr	y matter intak	te of con	centrate, f	orage, a	and total (co	ncentrat	e + forage)	) by die	et and period	l. Percen	t body wei	ight is	
calculated	oy dry matter	intake d	ivided by a	average	body weigh	nt from t	he respecti	ve peri	iod. Concent	trate and	forage dry	/ matte	r intakes
during LC	and HC diet v	vere diff	erent ( $P <$	0.0001	). Total dry	matter ir	ntake is rep	orted a	as the sum o	f concen	trate and fo	orage d	ry matter

Table 2-3. Dry matter intake of concentrate and forage

intake.

		Starch c	onsumpt	ion at 6	0 min	Starch co	nsumpti	on by n	ext meal	Tota	al starch	consum	led
		LC		1	HC	LC			HC	L		Н	IC
Period	BW	kg/meal	%BW	kg/d	%BW	kg/half- day	%BW	kg/d	%BW	kg/day	%BW	kg/d	%BW
1	395	0.12	0.03	0.67	0.17	0.12	0.03	0.71	0.18	0.25	0.06	1.41	0.36
2	403	0.14	0.04	0.66	0.16	0.14	0.04	0.71	0.18	0.29	0.07	1.42	0.35
3	407	0.14	0.04	0.59	0.15	0.14	0.04	0.69	0.17	0.29	0.07	1.38	0.34
Starch consu	mption cale	ulated basec	l off of r	efusals	as measur	ed by 60-mi	inutes po	ost mea	l, refusals b	y next mea	l, and to	tal daily	' starch
intake. Refus	als were no	ot measured i	for starcl	n conte	nt, estimat	ions based c	on starch	l conten	t determine	d in nutrier	nt analys	is. Perce	entage
of body weig	ht determin	ied by consu	mption (	(kg) div	rided by av	erage body	weight	(kg) fro	m each resj	pective peri	od.		

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## Table 2-5. Estimated dietary nutrient consumption

Estimated nutrient consumption on a daily basis on average DMI of both concentrate and hay

	DIETARY CON	SUMPTION
	LCHF	HCLF
CP, g	870	910
ADF, g	2,890	1,620
NDF <sup>3</sup> , g	4,430	2,570
Starch <sup>4</sup> , g	300	1,300
$WSC^5$ , g	870	900
$NSC^{6}, g$	1,180	2,190
ESC <sup>7</sup> , g	550	490
Crude Fat <sup>8, 10</sup> , g	120	170
NFC <sup>9</sup> , g	2,230	3,310
Ash <sup>10</sup> , g	180	260
Ca, g	80	60
P, g	30	30
Mg, g	30	20
K, g	110	90
Na, g	10	410

<sup>1</sup> DM basis, as analyzed by Dairy One Forage Lab (Ithaca, NY). <sup>2</sup> Acid detergent fiber: cellulose and lignin component.

- <sup>3</sup> Neutral detergent fiber: hemicelluloses, cellulose, and lignin component.
- <sup>4</sup> Starch: polysaccharide component.
- <sup>5</sup> Water soluble carbohydrate: monosaccharides, disaccharides, and some polysaccharides (fructans).
- <sup>6</sup> Non-structural carbohydrate: starch + water soluble carbohydrate.
- <sup>7</sup> Ethanol soluble carbohydrate: monosaccharides and disaccharides extracted in 80% ethanol.
- <sup>8</sup> Crude fat: determined by ether extraction.
- <sup>9</sup> Non-fibrous carbohydrate: an estimate of non cell wall carbohydrate. NFC = 100 (CP% + NDF% + Crude Fat % + Ash %).

<sup>10</sup>Values include concentrate consumption only, forage was not analyzed for this nutrient.

Urine	Average		%BW
Collection	BW	Forage Consumed (kg)	consumed
1	394	10.4	2.6
2	397	10.5	2.7
3	403	10.9	2.7
4	402	10.2	2.5
5	411	10.3	2.5
6	403	8.6	2.1

**Table 2-6.** Dry matter intake of hay during sugar absorption tests

Average forage consumption of hay during sugar absorption tests, day 14 of each period. Animals were fed a total of 2 kg (as fed) every 4 hours, beginning two hours after sugar administration. Total hay fed over the 24-hour urine pool was 12 kg (as fed). Total refusals were recorded at the end of the 24-hour test. A significant difference in intake was observed by period (P < 0.0001). Differences in least squares means revealed period 6 was different from all other periods (P < 0.0001) and intake during periods 3 and 4 were different (P = 0.0275). All other period comparisons did not reveal significant differences (P > 0.1000).

Table 2-7. Percent excre		or or all dose	or sugar ma	rkers during	101a1 24-110	u sugar aoso	orbrion test,	suppiement	companson
Sugar Marker		Percent I	Excretion of	oral dose	P-value	Percent I	Excretion of	oral dose	P-value
Ő	n	WBC	CBC	NONE	Diet	Period 1	Period 2	Period 3	Period
Sucrose	27	$4.8\pm0.8$	$4.3\pm0.8$	$4.6\pm0.8$	0.7837	$3.6\pm0.8$	$4.7\pm0.8$	$5.4\pm0.8$	0.1959
Lactulose	27	$2.2\pm0.4$	$2.1\pm0.5$	$2.3\pm0.5$	0.9419	$2.3\pm0.5$	$2.2\pm0.5$	$2.1 \pm 0.5$	0.9889
Mannitol	27	$3.4\pm0.3$	$3.0\pm0.3$	$3.0\pm0.3$	0.2099	$3.0\pm0.3$	$3.0\pm0.3$	$3.4\pm0.3$	0.4041
Sucralose	27	$2.4\pm0.2$	$2.4 \pm 0.2$	$2.7\pm0.2$	0.4698	$2.5\pm0.2$	$2.6 \pm 0.2$	$2.4 \pm 0.2$	0.5096
Lactulose: mannitol	27	$0.6\pm0.1$	$0.7\pm0.1$	$0.8\pm0.1$	0.3806	$0.7\pm0.1$	$0.7\pm0.1$	$0.6\pm0.1$	0.7533
Sucralose: mannitol	27	$0.7\pm0.1$	$0.8\pm0.1$	$0.9\pm0.1$	0.0946	$0.9\pm0.1$	$0.9 \pm 0.1$	$0.7\pm0.1$	0.1547
Percent excretion of oral	l dose	: (milligram	s of sugar e	xcreted over	respective	oool)/(millig	rams of suga	ar orallv adm	inistered).

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Sugar Marker		Percent I	Excretion of	oral dose	P-value	Percent E	Excretion of	oral dose	P-value
0	n	WBC	CBC	NONE	Diet	Period 1	Period 2	Period 3	Period
Sucrose	18	$1.1 \pm 0.1$	$0.4\pm0.1$	$0.7\pm0.2$	0.0543	$0.4 \pm 0.2$	$1.0 \pm 0.2$	$0.9\pm0.2$	0.0425
Lactulose	26	$0.4\pm0.1$	$0.3\pm0.1$	$0.2 \pm 0.1$	0.4055	$0.2\pm0.1$	$0.4 \pm 0.2$	$0.4\pm0.1$	0.3886
Mannitol	25	$1.6 \pm 0.3$	$1.3\pm0.2$	$1.2 \pm 0.3$	0.3648	$1.2\pm0.3$	$1.3\pm0.3$	$1.5\pm0.2$	0.5078
Sucralose	25	$0.7\pm0.1$	$0.5\pm0.1$	$0.5\pm0.1$	0.3273	$0.5\pm0.1$	$0.5\pm0.1$	$0.7\pm0.1$	0.2973
actulose:mannitol <sup>1</sup>	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ucralose: mannitol	25	$0.4 \pm 0.1$	$0.4 \pm 0.0$	$0.5 \pm 0.1$	0.5100	$0.4\pm0.1$	$0.4 \pm 0.1$	$0.5\pm0.0$	0.420-

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Sugar Marker		Percent	Excretion of	oral dose	P-value	Percent ]	Excretion of c	ral dose	P-value
0	n	WBC	CBC	NONE	Diet	Period 1	Period 2	Period 3	Period
Sucrose	23	$0.9\pm0.15$	$0.4 \pm 0.16$	$0.6\pm0.15$	0.1856	$0.5\pm0.15$	$0.8\pm0.14$	$0.6 \pm 0.14$	0.3865
Lactulose	21	$0.3\pm0.05$	$0.2\pm0.06$	$0.2\pm0.05$	0.2912	$0.2\pm0.05$	$0.3 \pm 0.06$	$0.2\pm0.04$	0.3218
Mannitol	23	$0.7\pm0.08$	$0.4\pm0.09$	$0.6\pm0.08$	0.0732	$0.5\pm0.08$	$0.7\pm0.09$	$0.5\pm0.08$	0.3613
Sucralose	22	$0.5\pm0.04$	$0.3\pm0.04$	$0.4\pm0.04$	0.0457	$0.3\pm0.04$	$0.4\pm0.04$	$0.3 \pm 0.04$	0.2702
Lactulose: mannitol	21	$0.4\pm0.05$	$0.4\pm0.07$	$0.4\pm0.05$	0.6064	$0.4\pm0.05$	$0.4\pm0.06$	$0.5 \pm 0.05$	0.4995
Sucralose: mannitol	22	$0.6\pm0.07$	$0.7 \pm 0.06$	$0.6 \pm 0.06$	0.5456	$0.7 \pm 0.06$	$0.7 \pm 0.06$	$0.6 \pm 0.06$	0.649

**Table 2-9.** Percent excretion of oral dose of sugar markers during 7 to 12 hour sugar absorption test, supplement comparison

Sugar Marker		Percent I	Excretion of	oral dose	P-value	Percent I	Excretion of	oral dose	P-value
0	n	WBC	CBC	NONE	Diet	Period 1	Period 2	Period 3	Period
Sucrose	25	$2.1 \pm 0.6$	$1.9\pm0.5$	$2.0\pm0.5$	0.9793	$1.7\pm0.5$	$1.8\pm0.5$	$2.4 \pm 0.6$	0.653
Lactulose	26	$0.3 \pm 0.1$	$0.3 \pm 0.1$	$0.3\pm0.1$	0.9010	$0.3\pm0.1$	$0.3 \pm 0.1$	$0.3 \pm 0.1$	0.7507
Mannitol	26	$0.5\pm0.1$	$0.5\pm0.1$	$0.5\pm0.1$	0.9948	$0.6\pm0.1$	$0.4\pm0.1$	$0.5\pm0.1$	0.4616
Sucralose	25	$0.4\pm0.1$	$0.5\pm0.1$	$0.5\pm0.1$	0.9533	$0.5\pm0.1$	$0.5\pm0.1$	$0.4\pm0.1$	0.4784
Lactulose: mannitol	26	$0.6\pm0.1$	$0.7\pm0.1$	$0.6\pm0.1$	0.8246	$0.6\pm0.1$	$0.7\pm0.1$	$0.7\pm0.1$	0.3077
Sucralose: mannitol	25	$1.0 \pm 0.1$	$1.0 \pm 0.1$	$1.0 \pm 0.1$	0.9279	$0.9\pm0.1$	$1.2 \pm 0.1$	$0.9\pm0.1$	0.0095

Table 2-10. Percent excretion of oral dose of sugar markers during 13 to 18 hour sugar absorption test, supplement comparison

Sugar Marker		Percent ]	Excretion of (	oral dose	P-value	Percent I	Excretion of c	oral dose	P-value
c	n	WBC	CBC	NONE	Diet	Period 1	Period 2	Period 3	Period
Sucrose	25	$2.1\pm0.70$	$2.5\pm0.66$	$2.6\pm0.63$	0.7944	$1.9\pm0.65$	$2.9\pm0.70$	$2.5\pm0.63$	0.2269
Lactulose	26	$0.3 \pm 0.06$	$0.4\pm0.05$	$0.4\pm0.05$	0.6776	$0.4\pm0.05$	$0.4\pm0.06$	$0.3\pm0.05$	0.5741
Mannitol	26	$0.4\pm0.06$	$0.5\pm0.05$	$0.5\pm0.05$	0.9438	$0.4\pm0.05$	$0.5\pm0.05$	$0.4 \pm 0.05$	0.5605
Sucralose	25	$0.4\pm0.06$	$0.4\pm0.06$	$0.4\pm0.07$	0.837	$0.4\pm0.06$	$0.5\pm0.07$	$0.3\pm0.07$	0.0915
Lactulose: mannitol	26	$0.7\pm0.04$	$0.9\pm0.04$	$0.9\pm0.04$	0.0077	$0.8\pm0.04$	$0.8\pm0.04$	$0.8\pm0.04$	0.853
Sucralose mannitol	25	$0.9 \pm 0.10$	$0.9\pm0.09$	$0.9 \pm 0.10$	0.9931	$0.9\pm0.09$	$1.0 \pm 0.10$	$0.8 \pm 0.10$	0.1233

Table 2-11 Dercent excretion of oral dose of sugar markers during 19 to 24 hour sugar absorption test supplement comparison

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		Percent E	xcretion of		Damaant			
		oral	dose	P-value				P-value
Sugar Marker	n	LCHF	HCLF	Diet	Period 1	Period 2	Period 3	Period
Sucrose	53	$2.3 \pm 0.4$	$4.7\pm0.4$	< 0.0001	$2.7\pm0.5$	$3.8\pm0.5$	$4.0 \pm 0.5$	0.0192
Lactulose	53	$1.7\pm0.3$	$2.2\pm0.3$	0.0444	$2.0 \pm 0.3$	$2.0\pm0.3$	$1.9\pm0.3$	0.8788
Mannitol	54	$2.4\pm0.2$	$3.1\pm0.2$	0.0009	$2.8\pm0.2$	$2.8\pm0.2$	$2.8\pm0.2$	0.9304
Sucralose	54	$2.3 \pm 0.2$	$2.5\pm0.2$	0.1233	$2.4 \pm 0.2$	$2.5\pm0.2$	$2.3 \pm 0.2$	0.7869
Lactulose: mannitol	53	$0.8\pm0.1$	$0.7\pm0.1$	0.4262	$0.7\pm0.1$	$0.7\pm0.1$	$0.8\pm0.1$	0.7469
Sucralose: mannitol	54	$1.0\pm0.1$	$0.8\pm0.1$	0.0524	$0.9\pm0.1$	$0.9\pm0.1$	$0.9\pm0.1$	0.9389

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		Percent Ex	ccretion of		Percent I	averetion of o	ral doce	
		oral	dose	P-value				P-value
Sugar Marker	n	LCHF	HCLF	Diet	Period 1	Period 2	Period 3	Period
Sucrose	28	$0.4\pm0.1$	$0.7\pm0.1$	0.0197	$0.4\pm0.0$	$0.5\pm0.1$	$0.6 \pm 0.1$	0.4619
Lactulose	25	$0.5\pm0.2$	$0.5\pm0.1$	0.9422	$0.6 \pm 0.2$	$0.5\pm0.2$	$0.6 \pm 0.2$	0.8655
Mannitol	48	$1.1 \pm 0.2$	$1.4 \pm 0.2$	0.258	$1.3\pm0.2$	$1.1 \pm 0.2$	$1.3\pm0.2$	0.6599
Sucralose	51	$0.4\pm0.1$	$0.5\pm0.1$	0.1636	$0.5\pm0.1$	$0.5\pm0.1$	$0.6\pm0.1$	0.6569
Lactulose: mannitol	25	$0.2\pm0.1$	$0.3 \pm 0.1$	0.3695	$0.3 \pm 0.2$	$0.3 \pm 0.1$	$0.1\pm0.1$	0.5895
Sucralose: mannitol	48	$0.9\pm0.4$	$0.4 \pm 0.4$	0.3385	$0.4 \pm 0.5$	$1.1 \pm 0.4$	$0.5\pm0.5$	0.4874

Table 2-13. Percent excretion of oral dose of sugar markers during 0-6 hours of sugar absorption test, dietary comparison

		Percent Excret	ion of oral dose	P-value	Percent	Excretion of o	ral dose	P-value
Sugar Marker	n	LCHF	HCLF	Diet	Period 1	Period 2	Period 3	Period
Sucrose	45	$0.5 \pm 0.10$	$0.7\pm0.09$	0.1153	$0.5 \pm 0.12$	$0.7 \pm 0.11$	$0.5 \pm 0.11$	0.2261
Lactulose	43	$0.3\pm0.05$	$0.3\pm0.05$	0.9002	$0.3\pm0.05$	$0.3\pm0.05$	$0.2\pm0.05$	0.4396
Mannitol	47	$0.6\pm0.09$	$0.6\pm0.09$	0.8815	$0.6 \pm 0.10$	$0.7\pm0.10$	$0.6 \pm 0.10$	0.6001
Sucralose	48	$0.4\pm0.04$	$0.4\pm0.05$	0.6194	$0.4 \pm 0.05$	$0.4\pm0.05$	$0.4\pm0.05$	0.5556
Lactulose: mannitol	42	$0.4\pm0.03$	$0.4\pm0.03$	0.8534	$0.4 \pm 0.04$	$0.4\pm0.04$	$0.4\pm0.04$	0.5988
Sucralose: mannitol	46	$0.7\pm0.04$	$0.7\pm0.04$	0.8901	$0.7\pm0.04$	$0.6\pm0.05$	$0.7\pm0.05$	0.7534

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		Percent Excre	tion of oral dose	P-value	Percent	Excretion of c	oral dose	P-value
Sugar Marker	n	LCHF	HCLF	Diet	Period 1	Period 2	Period 3	Period
Sucrose	49	$1.8 \pm 0.35$	$2.0 \pm 0.35$	0.5956	$1.8\pm0.38$	$1.7\pm0.40$	$2.0 \pm 0.38$	0.7779
Lactulose	50	$0.2\pm0.04$	$0.3\pm0.04$	0.0639	$0.3\pm0.04$	$0.3\pm0.04$	$0.3\pm0.04$	0.7993
Mannitol	51	$0.4\pm0.05$	$0.5\pm0.05$	0.0769	$0.5\pm0.06$	$0.4\pm0.06$	$0.4\pm0.06$	0.3463
Sucralose	50	$0.4\pm0.04$	$0.5\pm0.04$	0.2136	$0.5\pm0.05$	$0.4\pm0.05$	$0.4\pm0.05$	0.2558
Lactulose: mannitol	50	$0.6\pm0.04$	$0.6\pm0.04$	0.1277	$0.6\pm0.05$	$0.6\pm0.05$	$0.6\pm0.05$	0.9618
Sucralose: mannitol	46	$1.1\pm0.09$	$1.0\pm0.09$	0.0641	$1.0\pm0.09$	$1.1\pm0.09$	$1.1\pm0.09$	0.3402

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		Percent Excreti	ion of oral dose	P-value	Percent ]	Excretion of o	ral dose	P-value
Sugar Marker	n	LCHF	HCLF	Diet	Period 1	Period 2	Period 3	Period
Sucrose	49	$2.3\pm0.48$	$2.4 \pm 0.48$	0.8246	$1.6 \pm 0.51$	$2.8\pm0.51$	$2.5 \pm 0.52$	0.0262
Lactulose	51	$0.3\pm0.04$	$0.4\pm0.04$	0.0535	$0.3\pm0.04$	$0.4\pm0.04$	$0.3\pm0.04$	0.0496
Mannitol	51	$0.4\pm0.04$	$0.5\pm0.04$	0.1362	$0.4 \pm 0.04$	$0.5\pm0.04$	$0.4\pm0.04$	0.1263
Sucralose	49	$0.4\pm0.05$	$0.4\pm0.05$	0.7848	$0.4\pm0.05$	$0.5 {\pm}~ 0.05$	$0.4\pm0.05$	0.0191
Lactulose: mannitol	51	$0.8\pm0.04$	$0.8\pm0.04$	0.8836	$0.8\pm0.04$	$0.8\pm0.04$	$0.9\pm0.04$	0.6477
Sucralose: mannitol	50	$1.1 \pm 0.08$	$0.9 \pm 0.08$	0.0003	$1.0 \pm 0.08$	$1.1 \pm 0.08$	$1.0 \pm 0.08$	0.1069

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		STUDY LEN	GTH: 12 weeks		
PERIO	D 1: 28 d	PERIO	D 2: 28 d	PERIO	D 3: 28 d
14 d	14 d	14 d	14 d	14 d	14 d
LCHF WASHOUT INTERVAL 1	HCLF TREATMENT INTERVAL 2	LCHF WASHOUT INTERVAL 3	HCLF TREATMENT INTERVAL 4	LCHF WASHOUT INTERVAL 5	HCLF TREATMENT INTERVAL 6

# Figure 2-1. Visual description of full study design

The entire study was 84 days in length, with three 28 d periods. Each period consisted of one 14 d low concentrate high forage washout interval and a 14 d high concentrate low forage treatment interval.

						INT	ERV	AL (	14 d)					
Day in Interval	1	2	3	4	5	6	7	8	9	10	11	12	13	14

**Figure 2-2.** Visual description of sampling protocol within each of the 14-day intervals Rectal body temperature was recorded twice daily. Concentrate refusals were recorded 60 minutes post meal and prior to following meal. Hay refusals were recorded prior to following meal. Packed cell volume and total plasma sampling occurred during the seventh day of each interval, as indicated by the grey shading. Fecal pH and body weight were recorded at both the seventh and fourteenth days of each interval. Sugar absorption tests took place on the final day of each 14-day interval, as indicated by the black shading.

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Figure 2-3. Visual description of urine collection pools during sugar absorption tests

samples, 6-hour urine collections were pooled and sampled. sample was preserved with 10% thymol in non-dilute isopropanol. In addition to the individual and complete 24-hour urine The sugar absorption tests required a 24-hour complete urine collection. The volume of each individual urination was recorded and



## Figure 2-4. Dietary effects on fecal pH

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Average fecal fluid pH by diet, presented as means  $\pm$  standard deviation. LCHF was significantly different by diet from HCLF (*P*<0.0001).



Figure 2-5. Supplemental comparison of percent excretion of oral dose of sucrose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of sucrose excreted over the respective time point divided by the amount of sucrose administered at the start of the sugar absorption test (28.0 g). If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-6. Supplemental comparison of percent excretion of oral dose of lactulose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of lactulose excreted over the respective time point divided by the amount of lactulose administered at the start of the sugar absorption test (28.0 g). If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-7. Supplemental comparison of percent excretion of oral dose of mannitol

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of mannitol excreted over the respective time point divided by the amount of mannitol administered at the start of the sugar absorption test (16.0 g). If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-8. Supplemental comparison of percent excretion of oral dose of sucralose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of sucralose excreted over the respective time point divided by the amount of sucralose administered at the start of the sugar absorption test (80.0 g). If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-9. Dietary comparison of percent excretion of oral dose of sucrose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of sucrose excreted over the respective time point divided by the amount of sucrose administered at the start of the sugar absorption test (28.0 g). Values shown for HCLF diet are pooled averages of supplemental groups. If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-10. Dietary comparison of percent excretion of oral dose of lactulose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of lactulose excreted over the respective time point divided by the amount of lactulose administered at the start of the sugar absorption test (28.0 g). Values shown for HCLF diet are pooled averages of supplemental groups. If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-11. Dietary comparison of percent excretion of oral dose of mannitol

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of mannitol excreted over the respective time point divided by the amount of mannitol administered at the start of the sugar absorption test (16.0 g). Values shown for HCLF diet are pooled averages of supplemental groups. If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \le 0.05$ ).



Figure 2-12. Dietary comparison of percent excretion of oral dose of sucralose

Percent excretion is presented as mean  $\pm$  standard error and was calculated by the milligrams of sucralose excreted over the respective time point divided by the amount of sucralose administered at the start of the sugar absorption test (80.0 g). Values shown for HCLF diet are pooled averages of supplemental groups. If a statistical trend or significance was detected in the overall test of fixed effects of supplement, then adjusted *P*-values from individual differences of least square means were reported. Superscripts that differ within urine pool indicate a statistical difference (adjusted  $P \leq 0.05$ ).

#### **CHAPTER 3: Further Implications**

The work presented within this thesis provides preliminary insight into the optimization and use of the sugar absorption test as a measure of changes in gastrointestinal permeability in the horse. While these techniques have been extensively utilized over the past few decades in human medicine, much is still unknown about the different factors that influence the rates of absorption, fermentation, renal clearance, and the impact of osmolality among other factors. The larger disaccharides, sucrose, lactulose, and sucralose, only cross from the lumen of the gastrointestinal tract to circulation at the point of cellular or junctional damage. The mechanisms of the only administered monosaccharide, mannitol, are less understood, however it is thought that it serves as a correction factor for differences in surface area and water absorption. The preparation that is necessary in the identification of these markers is complex, however with more recent technologies the sensitivity of this technique is worth pursuing. Based on the results observed within this project, the investigation of research models including starch and fructan consumption with particular pertinence to laminitis, ulceration, and equine metabolic syndrome is worthy of consideration and will be discussed below.

### **Dietary induced increased permeability**

The working hypothesis of laminitis models involves a variety of unresolved mechanisms. While the application of both starch and fructan overload has been clearly validated within the horse, these models do not provide a practical scenario as to the majority of laminitis cases (Garner et al., 1975; van Eps and Pollitt, 2006). Further, it is known that the administration of these materials to the equine results in laminitic changes, but a vast majority of the mechanisms have yet to be elucidated.

As hypothesized by Clarke et al., the consequence of feeding high starch or fructans diets increases the risk of barrier damage within the gastrointestinal tract and an associated immune response (1990). A clear distinction of cecal contents of animals on high and low starch diets was observed and the conditions were likened to that of frothy bloat in ruminants (Lopes et al., 2004). The appearance of circulatory endotoxin following a subacute ruminal acidosis grain challenge within the dairy cow has been shown on numerous occasions (Motoi et al., 1992; Gozho et al., 2007; Li et al., 2012). This has been assumed as a measure of increased permeability. The quantification of appearance of a known marker within both the equine and bovine model would be beneficial in support of this theory. A more recent review has hypothesized fructan consumption to increased permeability and lactic acidosis at high and low levels of consumption (Johnson et al., 2013.) Fructanases, enzymes capable of degrading fructans, are possessed by various species under the Firmicute phylum. In mice models, fructose has been linked to increased gastrointestinal transit of endotoxin as well (Bergheim et al., 2008). The function of the fructan in increasing permeability lies in both the production of fructose for its involvement in cellular ATP depletion (van den Berghe et al., 1977; Cirillo et al., 2009) and its involvement in reducing pH when rapidly fermented (van Eps and Pollitt, 2006).

### Future directions for sugar absorption tests in the horse

Many details regarding appropriate fast duration, dosage of sugar markers, preservation of urine, and adequate detection of sugar markers were identified during this thesis work. However there are also many details that if modified would provide greater ease and success of this technique for the use in the horse. For the purposes of future application both in and outside of research settings, it is critical that this test be validated for use in plasma samples. The necessity of complete urine collection from each horse required very close monitoring of all animals at all times throughout the entire 24-hour collection period, in addition to constant maintenance of the urine collection devices. The mares were more difficult to achieve complete collections on and thus the success of complete collection devices used in this research setting would not be appropriate for use on a privately owned animal, as it required Velcro strips be glued onto the backside of each mare. These strips stayed on for several months and tended to remove hair if removed by other horses in the pasture. The utilization of plasma sampling would eliminate much of the variation in urine volume and preparation involved in conducting one of these tests in the horse.

The best option moving forward would be to conduct a 24-hour study in which frequently sampled plasma samples were acquired, allowing for the mathematical modeling of excretion into circulation. This application would provide a more precise identification as to the time course and pattern of marker excretion. The horse is a good model for this frequent plasma sampling, as these frequent blood samples would not endanger the animals due to their large blood volume and frequent samples could be taken to allow for a clear excretion profile. The utilization of modeling has not been employed within the field of sugar absorption tests and may even provide further insight in the human field.

In consideration of the sugar markers utilized, cost was not prohibitive despite the large doses required in comparison to human models. Following the interpretation of our

data, it would be beneficial to be able to separate dietary sucrose from the marker utilized in our study. This is of concern with regard to the larger concentrations than expected during our later urine pools. In addition, following further progress within our lab it may be most beneficial to utilize a mass spectrometer for detection of sugar markers. This provides a specific identification and quantification of each individual compound within the sample. Another potential modification of this work would be to administer equal doses of each disaccharide to the horses at the start of the 24-hour SAT. This is contrary to the relative sugar doses utilized throughout the literature, however no clear justification for utilizing different dosages is provided. The sucralose dose utilized during this project had to be increased based on low detection levels when analyzed via GC-FID. Additionally, sucralose co-eluted with mannitol leading to further difficulty of separation at low concentrations. If GC-FID is to be used for analysis, modifying the dose of both mannitol and sucralose should be carefully considered.

The analysis of several additional supporting variables has been proposed based on the findings of this study. Increased concentrations of bacterial DNA and D-lactic acid in the plasma could potentially both serve as indications of increased translocation of luminal contents into the circulation. The detection and identification of an equine specific tight junctional protein could potentially be monitored for quantification of paracellular damage. With specific regard to the supplementation of the horse with bovine colostrum, the identification of a bovine specific immunoglobulin or other component of colostrum within both the plasma and feces would be valuable. The appearance of bovine specific component in the plasma would verify its translocation and potential utilization at the gastrointestinal lining. Contrastingly, the appearance of bovine specific components in the feces of the horse would verify that the supplement reached the hindgut of the horse for potential protective function.

### Conclusions

This thesis provides initial work into the utilization of sugar absorption tests within the horse and further implicates diets that are high in starch and low in forage increase gastrointestinal permeability in the horse. The field of equine nutrition and gastrointestinal disease may benefit from the use of this technique in elucidating the extent of damage of various conditions. Further investigation is warranted in order to determine appropriate reference ranges for excretion, to better identify of time course of excretion, and improve sample preparation and analysis techniques.

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#### APPENDICES

### **Appendix A: Assay Procedures and Validation**

#### Packed Cell Volume

Packed cell volume was evaluated via microcentrifuge within 60 minutes of jugular vein collection into a 12-ml potassium ethylenediaminetetraacetic acid (EDTA) BD vacutainer tube. HEMATO-CLAD® mylar wrapped hematocrit tubes were purchased from Drummond Scientific Company and utilized for processing.

#### Alditol Acetate Preparation

\*Acknowledgements of protocol to Keshavarzian Lab, Digestive Diseases and Nutrition at Rush University Medical Center

- Reagents: Trifluoroacetic acid (TFA), Ammonium hydroxide, sodium borodeuteride, DMSO, glacial acetic acid, 1-methylimidazole, acetic anhydride, methylene chloride, acetone
- Equipment: Heating block, blow-down apparatus, PYREX® 9mL Screw Cap Culture Tubes with PTFE Lined Phenolic Caps, 13x100mm (Product #9826-13), disposable glass Pasteur pipettes, 2.0ml amber GC vials, gas chromatograph equipped for capillary columns and FID detector, a SPB-225 capillary column (Supelco) which was 30m×0.25mm I.D. column, with a 0.25µm film thickness.
- \*All materials purchased from Sigma Aldrich.

Initially, culture tubes are labeled for sample identification purposes. 100ul of urine sample is added to tube following thorough vortexing. Sample is then to be dried under nitrogen. To hydrolyze the sample, 250ul of 2N TFA is added to the dry sample, capped, and heated for 60 minutes at 121°C. Following the 60 minute heat period, the sample will again be dried under nitrogen. To further remove excess TFA, 200ul of isopropanol, dry with nitrogen, and repeat 1X. In order to reduce the sample, 100ul of 1M ammonium hydroxide is then added to the tube. 0.5ml of 20 mg NaBD<sub>4</sub>/ 1.0 ml DMSO solution made earlier is added to the tube and capped. The capped tube is then heated for 90 minutes at 40°C. Allow samples to cool to room temperature and then add 5-7 drops of acetic acid (samples will bubble upon addition of acetic acid). Then add 100 microliters of 1-methylimidazole to each tube. 0.5ml of acetic anhydride should be added to the solution and vortexed. 0.5ml of acetic anhydride is added and vortexed and allowed to stand for 10 minutes. 4ml of distilled water and 1 ml of dichloromethane are then added and vortexed to mix thoroughly. The sample is then centrifuged at 1500rpm and dichloromethane layer will separate from the water layer. The dichloromethane layer is removed and another 1.0ml dichloromethane is added to the original tube, vortexed, and centrifuged again. The second dichloromethane layer is added to the original layer that had been removed. The 2.0ml dichloromethane layers are then dried under nitrogen. The dried sample is then dissolved in 0.5ml acetone and added to the 2.0ml GC vial for analysis.
The detector temperature was 300° C and the injector temperature was 240° C. The initial column temperature of 100° C was held for 2 min and then increased at a rate of 10° C/min to 180° C, which was held for 2 min and then increased at a rate of 4° C/min to 240° C, which was maintained for 15 min. The total run time was 42 min. Chromatographic analyses were performed on an Agilent GC-FID 7890.

Specific flow rates at the FID were 45 ml/min of  $H_2$ , 350 ml/min of air flow, and  $N_2$  flow was set to flow at a constant 25 ml/min. Details regarding the inlet include the heater set at 240° C, pressure at 14.5 psi, total flow rate 41 ml/min, and the septum purge flow rate of 20 ml/min. The inlet was set for split mode at a ratio of 20:1.

Specific retention times of each compound are dependent upon each of the above listed details, as well as column length, deterioration of internal lining (based on the number of samples analyzed on it), and peak size of compounds. Listed are retention times of the four sugar markers of interest, however the specific retention time did shift over the analysis period. These numbers are to be utilized as a general reference: mannitol (29.029 min), sucralose (29.181 min), lactulose (29.708 min), and sucrose (30.317 min).



Best-fit values	
Slope	25578 ± 471.1
Y-intercept when X=0.0	71.46 ± 25.74
X-intercept when Y=0.0	-0.002794
1/slope	3.910e-005
95% Confidence Intervals	
Slope	24367 to 26789
Y-intercept when X=0.0	5.292 to 137.6
X-intercept when Y=0.0	-0.005560 to -0.0002007
Goodness of Fit	
R square	0.9983
Sy.x	51.69
Is slope significantly non-zero?	
F	2948
DFn, DFd	1.000, 5.000
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	7
Maximum number of Y replicates	1
Total number of values	7
Number of missing values	3

# Mannitol Standard Curve in Urine Blank



# Sucrose Standard Curve in Urine Blank



# Lactulose Standard Curve in Urine Blank



Best-fit values	
Slope	3455 ± 126.5
Y-intercept when X=0.0	2.724 ± 3.749
X-intercept when Y=0.0	-0.0007884
1/slope	0.0002894
95% Confidence Intervals	
Slope	3104 to 3807
Y-intercept when X=0.0	-7.684 to 13.13
X-intercept when Y=0.0	-0.004105 to 0.002080
Goodness of Fit	
R square	0.9947
Sy.x	6.583
Is slope significantly non-zero?	
F	745.9
DFn, DFd	1.000, 4.000
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	6
Maximum number of Y replicates	1
Total number of values	6
Number of missing values	4

## **Appendix B: Supplemental ingredient composition**

38 DYNA K-S3, 5.350

108 SOYBEAN OIL, 5.332

Kalmbach Feeds Inc., Custom Mixer Pellet included in high concentrate diet

CFC/Concept5 Date: 09/21/12 Plant: 1 KALMBACH FEEDS INC. User: DBURKE Product: 913PA MIXER PL FOR T913PA TRIAL Page: 1 Batch: 2000.34 Nutr Class: 3 Ingr Costs: Owning Status: FEASIBLE \_\_\_\_\_ Trial Formula Cost: 407.72/Ton 20.39/Clb 0.2039/Lb Version: 1 **INGREDIENT SOLUTION: (Unrounded)** -----IngrCode, Ingredient Name, Amount Lbs \_\_\_\_\_ 1209 NEW HORSE 2.0 TM, 3.983 26 CORN, FINE GRIND, 835.78 107 SOYBEAN MEAL 46., 486.68 480 ZMC 842 (ALTECH), 2.985 71 STARCH, 407.86 891 INTEGRAL/MTB100, 2.729 51 CORN, DIST DR GR, 58.54 70 MAG OX 50% SCL 2, 2.131 123 WHEAT FLOUR, 11.5, 51.71 345 VIT E 50% SCALE3, 0.6471 121 WHEAT MIDDS, 15.8, 40.13 292 SELENIUM .20%, 0.6297 14 BIOPHOS SCALE 1, 33.17 359 ANISE OIL STRAIG, 0.6000 60 LIME-CALCIUM-SC, 26.52 254 STABL C 31%, 0.5350 2 ALFALFA DHY 17%, 17.05 675 LEVUCELL SC10ME, 0.3411 101 SALT - SCALE #1, 10.34 240 K-VITAMIN A-30, 0.2388 1210 NEW 5.0 HORSE VI, 6.822 250 K-VIT D-3 9KICU, 0.1280

1419 Alkosel 3000 – O, 0.1137

140

#### Supplemental nutrient composition

Kalmbach Feeds Inc., Custom Mixer Pellet included in high concentrate diet

### NUTRIENT SOLUTION:

No. Nutrient Name, Units, Analysis, Minimum, Maximum

\_\_\_\_\_ 3 CR PROTEIN, PCT 16.207 15.800 4 CRUDE FAT, PCT, 2.733, 2.734 5 CRUDE FIBER, PCT, 2.437 6 CALCIUM, PCT, 0.901, 0.901, 0.910 7 PHOS-TOTAL, PCT, 0.682, 0.682 9 ADF, PCT, 4.331 10 NDF, PCT, 8.287 15 TDN RUMINENT, PCT, 76.718 16 NET ENGAIN, MGCCWT, 58.513 18 NET ENLACT MGCCWT, 79.193 19 NET EN<sub>MAINT</sub> MGCCWT, 86.151 20 RU SOL PROTEIN, PCT CP, 4.293 22 NON-PROT NITROGE PCT, 0.000 41 DRY MATTER, PCT, 89.369 44 SALT, PCT, 0.572, 0.572, 0.580 45 ASH, PCT, 6.337 47 VITAMIN A, KIU/LB, 17.995 49 VITAMIN D-3, KIC/LB, 0.973 50 VITAMIN E, MG/LB, 264.3 62 SODIUM, PCT, 0.248 63 POTASSIUM, PCT, 0.871, 0.870 64 MAGNESIUM, PCT, 0.201, 0.201

65 SULFUR, PCT, 0.221, 0.221 66 SELENIUM, PPM, 0.846 67 CHLORIDE, PCT, 0.480 76 UIP-UNDEG IN-PRO PCT, 5.740 77 DIP-DEG IN-PROT, PCT, 11.605 78 NFC-NON FIB CARB %, 54.710 84 COPPER, PPM, 112.427, 112.413 85 IODINE, PPM, 1.546 86 IRON, PPM, 368.578, 368.566 87 MANGANESE, PPM, 178.298 88 ZINC, PPM, 314.010, 313.990 115 CHROMIUM, PPM, 0.000 138 STARCH % DM, 48.320 167 ORGANIC SEL, PPM, 0.170, 0.171 169 BOVATEC, G/TON, 0.000 170 RUMENSIN, G/TON, 0.000 171 CTC, G/TON, 0.000 180 DECCOX, G/TON, 0.000 189 dcad, meq/kg, meq/kg, 57.497 190 TYLAN, G/TON, 0.000 210 Butyrate, %, 0.000