Effect of Dietary Manipulation on Physiological Responses in Quarter Horses

A Thesis

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By
Jessica L. Saul
Graduate Program in Animal Sciences
The Ohio State University
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Thesis Committee:
Dr. Kimberly Cole, Adviser
Dr. Naomi Botheras
Dr. Michael Day
Dr. Frank Welker
ABSTRACT

Today’s horse management practices often include restricted access to forage and feeding large quantities of grains in a limited number of meals throughout the day. These practices may create psychological and physiological stress in the horse, influencing behavior and disrupting glucose and insulin pathways, the balance of microflora in the gastrointestinal tract, and immune responses. The studies presented in this thesis investigated the effects of dietary manipulation on physiological and behavioral responses in Quarters Horses in varying stages of production. In the first study, feeding frequency (1, 2 or 3 meals/d) and order of diet (grain fed before hay or vice versa) were varied to determine their influence on glucose, insulin and cortisol responses as well as stereotypic behaviors in Quarter Horse mares. The results from this study showed that while glucose, insulin, and cortisol concentrations varied with time (P < 0.0001), there were no differences due to feeding frequency or order. No differences were observed in crib-biting or pawing; however, horses fed 1 meal/d with grain given before hay were observed weaving more often than horses fed 2-3 meals/d (P < 0.05). In the second study, a commercial probiotic containing *E. faecium*, *L. acidiophilus*, *L. casei*, and *L. plantarum* was orally administered to young Quarter Horses over a period of 11 weeks to examine the effects on cortisol concentrations pre- and post-transport and antibody response to tetanus vaccination. There were no differences in tetanus antibody titers due to probiotic supplementation. In addition, supplementing a horse’s diet with the probiotic did not affect plasma cortisol concentrations pre- or post-transport when compared
between treatment groups; however, there was a significant increase in plasma cortisol concentrations within the control group (P < 0.05). Although probiotic supplementation may be beneficial to the overall health of the horse, specific effects related to stress and immune responses were not observed in this study. Overall, manipulating a horse’s diet through feeding frequency, the order in which hay and grain are fed, or probiotic supplementation were not shown to affect physiological or psychological responses of Quarter Horses in varying stages of production.
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VITA

July 14, 1987………………………Born - Columbus, Ohio

June 2005…………………………The Wellington School, Upper Arlington, Ohio

May 2009…………………………..B. S., Biology, Denison University

2010 – Present……………………Graduate Research Assistant, Department of

Animal Sciences, The Ohio State University

Publications


Field of Study

Major Field: Animal Sciences
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CHAPTER 1 – LITERATURE REVIEW

Introduction

Horses evolved to eat small and frequent meals throughout the day and will spend 16-18 hours per day grazing if given the opportunity (Youket et al., 1985). Today’s horse management practices often include restricted access to forage and feeding large quantities of grains in a limited number of meals throughout the day. These practices may create psychological and physiological stress in the horse, influencing behavior and disrupting glucose and insulin pathways, the balance of microflora in the gastrointestinal tract and immune responses. In addition, boredom from infrequent meals may increase stereotypic behaviors such as weaving and crib-biting (Cooper et al., 2005). The activity levels of hormones, such as glucocorticoids, that have the ability to manipulate gastrointestinal microflora are important to metabolic and physiologic function. Early research has shown that these hormones can be easily altered due to stress (Ralston, 1979). Alterations in certain metabolic pathways due to stress can result in deviations in microflora diversity and density in the gut. Changing the composition of microflora by reducing the number of beneficial bacteria can then decrease the response of microflora to pathogens as well as the ability to signal an immune response (Abbas et al., 2007). Although the mechanisms are not clearly defined, inflammatory cytokines, norepinephrine, or extra glucocorticoids are thought to play a role (Abbas et al., 2007).

Horses are exposed to many stressors in their daily routine including feeding management and transport (Swanson, 2002). When horses experience stress, their
metabolic system may overcompensate with production of cortisol in an attempt to protect the tissues from damage. An outcome of this can be the underproduction or overproduction of specific hormones such as glucose and insulin that bring energy to the cells and microflora in the body (Abbas et al., 2007). When the stress continues and cortisol is not repressed by normal feedback mechanisms, changes in the microflora can occur as well as a decrease in the efficiency of immune responses (Blum et al., 2002). To reduce the impact of stress on physiological and metabolic parameters, multiple strategies have been introduced such as increasing the frequency in meals fed per day, changing the order of which a meal is given, or supplementing the diet with probiotics, to either limit the production of cortisol or prevent changes in physiological function when cortisol is produced.

**Dietary Manipulation of Horses**

**Meal Frequency**

Alterating the frequency in which meals are fed can disrupt homeostasis between glucose, insulin, and cortisol and lead to changes in the metabolic pathways of the horse. Youket et al. (1985) found that ponies fed only 1 meal/d, without enough time to complete the entire meal, had higher concentrations of glucose following the morning meal due to an increase in beta oxidation that would have taken place during fasting at night. In comparison, ponies that were fed 6 meals/d had significantly lower glucose concentrations and were able to consume their meal within the hour time allotted. Although insulin was not measured in this study, the authors hypothesized that increased glucose concentrations would be associated with increased insulin concentrations in
ponies that were fed less often. In another study where horses were given two isoenergetic meals of 2 meals/d versus 1 meal/d, there was an increase in glucose due to starch overload when the horse was fed only 1 meal/d (van Soest, 1994).

In contrast, Youket et al. (1987) found that increasing the number of meals decreased cortisol production and improved glucose uptake into the cells by insulin. This was exhibited through an increased secretion of insulin in ponies fed less frequently. Increased stress is also evident during anticipatory events which can result in decreased insulin (Power and Shulkin, 2008). Horses that are fed less often experience more stress. This stems from being fasted for an extended period, as well as anticipation of the next meal. Increased anticipation results in numerous metabolic changes, such as increased cortisol production, decreased glucose uptake into cells and decreased insulin secretion, which can negatively affect the health of the horse (Power and Shulkin, 2008).

Van Weyenburg et al. (2007) also found no difference in glucose concentrations in horses fed 1 or 3 meals/d. There was no limitation placed on the amount of time the horse had to consume the entire meal. Because of this, horses that received one large meal did not consume that meal in a short period of time, but instead spread the meal out throughout the entire day much like a pastured horse would do. Glucose concentrations did not spike, because there was not one large carbohydrate load coming into the system. For horses that received 3 meals/d, there was not a significant rise of glucose after the second meal before the morning meal. This study did not measure insulin but assumed that regardless of treatment, horses would not have different insulin concentration due to no differences found with glucose concentrations. Van Weyenburg et al. (2007) also
found no differences in cortisol concentrations due to feeding frequency. They believed that this was due to when blood measurements were taken or because horses were allowed to eat at leisure and were therefore not fasted for a long period.

When there is a decreased frequency of meals fed in a day, there is increased anticipation for meals and less priming of the metabolic system for incoming glucose (Power and Shulkin, 2008). This could cause influence the cephalic phase of digestion. During this phase, the vagus nerve stimulates the secretion of digestive hormones from various organs in the body. This includes the secretion of insulin from the pancreas. For horses that are fed only 1 meal/d, the large carbohydrate load from the meal will cause a large influx of glucose from starch due to fermentation. When glucose is released into the blood from fermentation, increased insulin above usual concentrations is then necessary for transport. If the horse becomes stressed, glucose uptake time will be further lengthened due to the rate and concentration of uptake of glucose that cells are able to endocytosis through glucose receptors associated with a decrease in the efficiency of the insulin pathway (Asplin et al., 2007). The cephalic phase of digestion can also be altered due to stress reducing the pre-meal secretion of insulin and further exacerbating a hyperglycemic state (Power and Shulkin, 2008).

In contrast to Youket (1985), horses that were fed 2 meals/d had higher concentrations of glucose and insulin after being exercised then horses that were fed 4 meals/d. These horses were fed at twice the maintenance energy requirement, with 36% of the diet being from grain (Jansson et al., 2006). When horses were fed 2 meals/d,
researchers believed this to be similar to a horse being feed deprived. This included having low insulin concentrations and altered glucose metabolism. This relationship was also found by Stull and Rodiek (1988) in which there was decreased glucose concentration in horses fed 2 meals/d in comparison to 1 meal/d. Although cortisol production was not measured in these studies, it has been noted that an increase in cortisol due to feed deprivation can reduce the effects of the cephalic response under the sympathetic nervous system (Power and Shulkin, 2008). When there is activation of the sympathetic nervous system under the cephalic phase, there is an increase in insulin resistance due to epinephrine binding to the hypothalamus (Abbas et al., 2007).

Although cortisol has not always been measured in past research dealing with feeding frequency or did not change significantly, the levels of glucose and insulin have been shown to be affected by diet. A horse or pony fed less often had higher concentrations of glucose in some cases (possibly based on the study design), than an animal fed less often. Because of this, one strategy to reduce stress and keep glucose and insulin within normal levels is through increasing the number of times a horse is fed a day.

Microbes are very susceptible to changes in the gut due to diet, when the composition of the diet changes, the diversity and composition of the microbes can decrease (Clarke et al., 1990). Feeding frequency can also affect the microflora of the gut due to meal size and starch intake. Horses fed less frequently take in more starch from their diet in a shorter amount of time. Because the hindgut contains microbes that are responsible for the digestion of indigestible fiber and starch, large starch diets can change
the composition of the resident flora. The production of VFAs from fermenters can be easily manipulated due to diet because the composition of bacteria changes in order to better facilitate fermentation (Bergman et al., 1990). Microbes that become more dominant in high starch environments such as *Lactobacillus*, can then produce more lactic acid. Changes in the pH in the lumen irritates the gut lining (Pagan, 1998) and also affects other bacterial species present in the hindgut through apoptosis.

**Meal Order**

A review of the existing literature yields little information on the effects of meal order on physiological and psychological responses in the horse. In one study, an increase in glucose was associated with feeding grain 4 h before hay was given in exercised horses (Pagan and Harris, 2010). When hay was fed simultaneously with grain or 2 h after grain there were smaller peaks in glucose concentration. Insulin concentrations in these horses followed a very similar pattern to that of glucose.

In another study, horses fed hay first had increased saliva production (Ellis et al., 2002). The researchers hypothesized that with increased lubrication there would be a faster passage rate overall by the time grain was ingested as the second component of the meal, allowing for grain and hay be fermented simultaneously, and resulting in large glucose peaks. In a study investigating a horse’s anticipation and motivation to receive hay or grain, researchers found that horses pressed a panel more often when the next food item to receive was grain rather than hay (Elia et al. 2010). Due to this finding, the
authors concluded that horses were more motivated and anticipated getting grain more than hay. Because motivation is linked to the cephalic phase of digestion, a more dynamic and extended cephalic phase would have occurred when fed hay first, and more insulin would have been released.

**Stress**

Stress is the reaction of the body to stimuli that disrupts normal physiological equilibrium (Rostagno, 2009). Stress can be grouped into three distinct categories: physiological stress, stressors that consist of a physical stimulus, and stressors that challenge cardiovascular homeostasis (Rostagno, 2009). The disruption of normal functioning as a consequence of stress can lead to changes defined through physiologic or metabolic changes. Typical stressors for horses are transportation, illness, and weaning (Swanson, 2002). Stress can also be produced through dietary manipulations (Youket et al., 1985; Stull et al., 1987).

A direct outcome of stress is the production of cortisol through CRH (corticotropin-releasing hormone) from the hypothalamus and ACTH (adrenocorticotropic hormone) from the pituitary. ACTH, when activated, secretes glucocorticoids to follow through with their various functions in the body including glucose metabolism, gluconeogenesis, and stimulation of fat breakdown for an alternative glucose source (Horton et al., 2005). In a normal situation, glucocorticoids provide negative feedback to prevent further CRH and ACTH secretion.
Stress does not always result in adverse health responses. Due to epinephrine production from the sympathetic nervous system, animals are primed for fight or flight (Horton et al., 2005). During this short period, there can be an enhancement of metabolic functions that could lead to a promotion of immune action to help the host for a short spurt of time before immune function is repressed. Cortisol is also beneficial in autoimmune diseases in which inflammation needs to be suppressed. By binding to glucocorticoid receptors on the surface of organs, cortisol and other glucocorticoids can be secreted as an antagonist against immune complexes in type III hypersensitivities (Abbas et al., 2007).

Prolonged stress however, may result in chronic immunosuppression and loss of normal metabolic functions (Abbas et al., 2007). This results when the negative feedback loop from ACTH is unresponsive resulting in increased cortisol production and depletion in the adrenal cortex and the hypothalamus (McCall et al., 1987). Glucocorticoids during periods of stress continue to be produced, thereby damaging tissues and cells of the body. Not only can glucocorticoids affect the gastrointestinal microflora, but glucocorticoids can also affect the immune response through the suppression of inflammation through a mechanism which is not well known (Khansari et al., 1990). This response will continue until the animal is removed from the stress or they become resistant to the stress turning off the pathway and decreasing glucocorticoids in the blood.

The adverse effects of stress can be assessed through a horse's change in behavior, hematological, and physiological changes (Fazio et al., 2003). Physiological measurements of stress can be measured through the interaction of systems involved and
the functionality of the system after exposure to the stress. Haemotological variables of stress are measured through changes in the blood such as increases in glucose in the blood due to stress. Cortisol has been validated as a measurement of stress in the horse (Fazio et al., 2003). As stress increases, the ability of the horse to cope and maintain homeostasis becomes more difficult.

**Dietary Stress**

Although horses are designed to consume smaller meals due to their digestive physiology (Hintz and Cymbaluk, 1994), it is not always possible for management to mimic optimal conditions. Increased cortisol production has been shown to be most exacerbated in fasted horses (Van Weyenburg et al., 2007). Donkeys fasted overnight had transient increases of cortisol that returned to baseline concentrations. Donkeys that were fasted for 3 d had a significant increase in cortisol over baseline levels for an extended period resulting in changes to hormonal pathways (Forhead and Dobson, 1997).

An increase in fasting can also increase anticipation to be fed, which can result in increased cortisol production and a decrease in health as measured by decreased glucose control and the regulation of meal size (Power and Shulkin, 2008). In exercised horses that were fasted versus grazing horses that were not exercised and allowed to eat freely, insulin concentrations were significantly lower in grazing horses (Pagan and Harris, 1999). These studies exemplify that the longer an animal is fasted, especially while seeing others eat, the more cortisol will be produced and the less insulin produced. Stull et al. (1988), found fluctuations in cortisol concentrations due to anticipation of the diet.
Increased anticipation, as found in the study, has proved to be a valid indicator of stress (Power and Shulkin, 2008). By limiting the meals that a horse is fed per day, there is an increase in fasting time leading to higher anticipation of being fed.

**Transport Stress**

Transportation is very common in management routines; and whether it is for a short period of time or an extended time, there can be an increase in stress. Stress has been shown to increase in horses just by getting into and being in the trailer (Bradshaw et al., 1996). In addition to being in the trailer, stress has been shown to increase during unloading, confinement, vibrations, changes in temperature and humidity, and food and water deprivation (Waran et al., 1995; Broom, 2005). This stress can decrease the ability of the horse to have efficient inflammatory responses as shown through an increase in the incidence of respiratory infections in horses after transport (Okiawa et al., 1995).

Stallions that were transported for short or long distances showed similar stress indicator responses both before and after transport. This was observed through increased ACTH (adrenocortical thyroid hormone) that is highly correlated with changes in endocrine responses (Fazio et al., 2008). This was further exemplified in horses that were being shipped in a commercial trailer. Although there was variation with individual responses to transport, on average horses lost 4% of their body weight (Stull, 1999).

There is also considerable individual variation in horses’ responses to trailer loading and unloading. Young animals tend to response more aggressively than older, more experienced animals (Fazio et al., 2003). This can be shown through ears being
pinned back as an initial sign of aggression or the lowering and extension of the head as it occasionally accompanies biting, striking, or kicking (Waring, 2003). Plasma cortisol and glucose were found to be higher during transit of inexperienced racehorses than in experienced racehorses (White et al., 1991). Fazio et al. (2003) looked at the relationship between temperament and stress. Calmer horses experienced less stress, and therefore maintained physiological status. More aggressive horses had more changes in their overall health due to transport (Fazio et al., 2003).

The measurement of physiological changes post-transport has been well characterized in the horse. Increased heart rate has been noted in horses post-transport (Broom, 2005; Fazio et al., 2008). In cattle, an increased heart rate has shown to be highly correlated with increased cortisol production (Lay et al., 1994). This was also shown in horses, in which the highest heart rate occurred near the end of transport (Schmidt et al., 2010). Changes in cortisol production due to transport are also associated with immunological changes. Horses that were exposed to prolonged transport experienced large changes to their immune response from stress (Stull et al., 2010). While transport increased neutrophils and white blood cell counts to increase inflammation, it decreased T-lymphocyte subpopulations which would stimulate antibody production. Normal T cell populations did not return to normal until 24 h after transportation (Stull et al., 2010).

**Stress and Hormones**

**Cortisol**
A direct outcome of stress is the production of cortisol through CRH from the hypothalamus and ACTH from the pituitary. Cortisol initially helps relieve the horse of stress by increasing glucose uptake into the cells to increase heart rate, blood pressure, and respiration to better enable a ‘flight or fight’ response (Abbas et al., 2007). During prolonged stress, the negative feedback loop that ACTH is responsible for becomes unresponsive, resulting in increased cortisol production and depletion in the adrenal cortex of the hypothalamus (McCall et al., 1987). Although cortisol is initially helpful, it ultimately decreases the efficiency of glucose metabolism and further reduces immune responses through a decrease in lymphocyte proliferation or change to microflora composition.

**Glucose**

Glucose, similar to cortisol, is in the family of glucocorticoids and follows the same circadian rhythm with higher concentrations in the morning and lower peaks as the day goes on. Cortisol is high, particularly in the morning before a meal is ingested, to allow for gluconeogenesis while glucose is high due to no starch being ingested overnight to maintain glucose levels (Hucklebridge et al., 1999). With prolonged stress, negative feedback mechanisms are disabled and glucose continues to be secreted along with cortisol. Increased glucose can affect microflora through anti-inflammatory cytokine production that can reduce the overall efficiency of phagocytosis and antibody production (Abbas et al., 2007).
Insulin

The cephalic phase of digestion occurs before food is ingested and influences the reaction of the body, through neuronal signaling, to incoming food (Powley and Berthoud, 1985). Depending on the type of food, and its sight, smell and taste, that stimulates the vagus nerve, there will be different amounts of digestive hormones secreted (Powley and Bethoud, 1985). During this period, enervation of the vagus nerve occurs which allows for signal transduction to produce digestive hormones to aid in digestion. The cephalic part of the digestive process has been shown to have an active role in the HPA axis and cortisol production (Benedict et al., 2005). When cortisol is produced from stress, the cephalic phase can be bypassed reducing the concentration of insulin prior to a meal. A reduction in insulin pre-meal can result in a hyperglycemic state (Teff, 2002).

Stress and Gut Microflora

The symbiotic relationship of microbes in the gut is not stable and can fluctuate due to stress and changes to the diet, and result in increased pathogenesis (Hintz and Cymbaluk, 1994). Changes in the environment can modify the pH and temperature, destroying microbes that the horse depends on for digestion (Respondek et al., 2007). When the composition of the diet changes and increases the levels of glucose and insulin, microbes in the gut change in both composition and function.

Sudden diet changes cause the type of microbes present to be unable to efficiently digest the fiber and instead die off. It is important to slowly change the diet of the horse
so that the microbes are more able to adapt to the new diet. The starch fraction that is indigestible by mammalian enzymes reaches the cecum at a partially digested state. Bacteria such as *Lactobacillus* thrive on starch, and therefore an increase of starch in the hindgut would allow for significant proliferation of these bacteria. An increase in *Lactobacillus* will cause a buildup of lactic acid in the cecum (Hintz and Cymabaluk, 1994). Increased lactic acid will decrease the pH of the environment, killing off the other bacteria present, and throw off the balance of the colonies of microbes. As these microbes die off, endotoxins are released and can enter the bloodstream and cause colic and other conditions. For a horse that does not experience a lot of stress, maintaining a stable population is easier, and a change in the diet or gut can be potentially tolerable. For horses that do experience a lot of stress, maintaining a stable population can be difficult, and a change in the diet or gut can be detrimental.

**Stress and Immune Responses**

During times of stress, glucocorticoids function to prevent overstimulation of an immune response. However, during prolonged stress feedback mechanisms can be inhibited, resulting in longer wound healing time, increased susceptibility to disease, and impaired responses to vaccinations (Petrovosky and Harrison, 1997). Small bouts of stress, demonstrated through acute exercise, have smaller effects on the immune response causing transient immunosuppression (Kendal et al., 1990). This was shown through slight reductions in neutrophil proliferation followed by a fast recovery time where neutrophil numbers returned to normal (Kendal et al., 1990). During bouts of high
intensity exercise, or prolonged stress, there is an abrupt change in lymphocyte proliferation, reducing CD4 cells which are responsible for initiating antibody production through B cells, as well as diminishing CD8 cells that target viruses (Snow et al., 1993). This was shown through reduction of neutrophil activity for an extended period of time as well as a decrease in phagocytic activity of macrophages. In the study, when the stress was removed or ended, there was a period of recovery in which there was increased neutrophils 4 h after a treadmill test in Thoroughbreds (Snow et al., 1993).

**Probiotics**

Probiotics are live microorganisms that can be given orally to influence the intestinal microflora to benefit the host, are another strategy to reduce stress from diet or other management practices that can create host stress (Teitelbaum and Walker, 2002). The use of probiotics in animals is based on the ability of probiotics to enhance immune function against pathogenesis during periods of stress (Fuller, 1989; Haghihi et al., 2006). During stress, microbial balance can decrease in composition and diversity. By adding supplements into the diet, the effects of stress on the microflora can be reduced. The interaction of the product with microflora in the gut, allow for probiotics changing the immune response in ways that are not fully understood. In general, cellular responses can be enhanced or antibody production increased based on the strain of the bacteria and the health of the host.

Probiotics were first used in animals in the 1970’s by incorporation into the feed to improve animal health and increase resistance against disease, in particular to prevent
diarrhea (Fuller, 1989). The transition from examining the effects of probiotics in humans to farm animals occurred due to the belief that there would be a further stimulation of the immune system during times of duress (Fuller, 1989). This was examined in animals that were reared in complete absence of antibodies and were considered to be very susceptible to disease. This situation could be amended by the supplementation of a probiotic which was believed to colonize the gut to a level that antibodies could be produced (Fuller, 1989).

**Use of Probiotics in Animal Production**

The effects of probiotics in several species, including chickens, pigs, and cattle, have been evaluated with mixed results (Fuller, 1989; Haghihi et al., 2006; Taras et al., 2006). Poultry research involving probiotics has demonstrated that fermentable feeds can be associated with the improvement of health. Although there are many probiotic species available on the market associated with an improvement of health, in chickens Lactic Acid Bacteria (LAB) have been examined with the most successes (Patterson and Burkhold, 2003). *Salmonella* colonization of chicks was reduced significantly in that only 43% of chicks given the probiotic were colonized with *Salmonella* (Fritts et al., 2000). This was in comparison to 100% of control chicks that had colonization of *Salmonella*.

Chickens have also been supplemented with a probiotic containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis*, to test if there are differences with specific antibody production against tetanus toxoid (Haghihi et al.,
This study found that probiotics were successful in increasing both the IgM and IgG response against tetanus toxoid in comparison to unsupplemented 1d old chicks. This response details that probiotics were able to promote the humoral immune function and increase proliferation of both non-differentiated B cells and IgG B cells as well. Those that were supplemented would not only be able to mount a more successful immune reaction to tetanus, but would also be more prepared if ever in contact with the antigen again, due to buildup of memory cells against tetanus.

Although the uses and outcomes of probiotic supplementation in horses have been examined, the results obtained show different outcomes. Administering probiotics during a chronic state of stress such as weaning has been investigated with little results (Swanson, 2002). Foals that were given a probiotic during weaning were found to have higher lactate concentrations overall with successful colonization of the LAB in the gut. However, the increased lactic acid produced from LAB did not confer a change in fecal pH or antibody production (Swanson, 2002).

Another study examining the effects of the immune response after clinically healthy horses were supplemented with a product found that some probiotic strains were antagonists to the host (Botha, 2011). Using certain LAB strains the study found that there were transient and acute changes associated with increased neutrophil and eosinophil numbers (Botha, 2011). The strains examined for use in the horse were considered to be non-virulent; however, it was believed that the reaction that the host had to the strain was due to an intolerance reaction and not an enhanced immune response.
One of the challenges in administering probiotics is determining the correct dosage amount to efficiently achieve optimum results without harming the animal.

Mares that were given *L. rhamnosus* GG failed to show successful colonization in the gut regardless of dose (Weese et al., 2002; Weese and Rosseau, 2005). Only with a high dose that was considered to be harmful to the horse if dosed over an extended period, was there colonization. Colonization was still considered to be sporadic. Due to the problems with dose responses, it was believed that probiotics worked more efficiently with a host that was immunocompromised (Weese and Rosseau, 2005). This conflicted with Parraga et al. (2008) who found no changes in colonization based on the health status of the horse. Horses given a probiotic to inhibit *Salmonella* shedding exhibited no differences in *Salmonella* shedding, hospitalization time, or incidence of diarrhea compared to horses that did not receive the probiotic regardless of the dose that horses received. It was believed due to the results in this study that probiotics might function differently with healthy horses.

Probiotics have also been studied in mares and foals. In one study, mares and foals that were dosed with *Lactobacillus pentosus* were more apt to have diarrhea than control foals associated with abnormal clinical signs (Weese and Rosseau, 2005). Although it was believed that probiotics would function better if the animal was immunocompromised, with this set of horses there was an obvious decrease in health. This result is not isolated to horses. Dosing probiotics in immunodeficient humans that have an autoimmune disease or inflammation has been shown to be more harmful than helpful (Wagner and Balish, 1998).
Probiotics and Immune Function

Probiotics have been shown to enhance immune function through three general ways: enhancement of phagocytosis, increased production of IgG and IgM antibodies IgM, and increased local antibody IgA in the gut (Fuller, 1989). Some probiotics are able to modulate the immune response through epithelial cells and M cells that can be presented to macrophages to modulate innate or adaptive function. Due to macrophage phagocytosis, macrophages can then function as antigen presenting cells to cause proliferation of B cells at a lower energy cost.

Although probiotics are supposed to enhance the immune response through IgG proliferation, there have been mixed results on the actual occurrence of this happening. Weese and Rosseau (2005) found that IgG was reduced when horses and foals that had diarrhea were supplemented with a probiotic. Increased incidence of diarrhea and decreased proliferation of IgG was associated with abnormal clinical exams. Chickens that were given a probiotic, had no significant enhancement of IgM or IgG. Instead, there was only proliferation of tetanus-specific antibodies when challenged with tetanus (Haghihi et al., 2006).

Administering probiotics during a chronic state of stress, such as weaning, has been investigated with mixed results. Foals that were given a probiotic during weaning were found to have higher lactate concentrations overall, but these concentrations were unable to confer a change in fecal pH or antibody production of IgA and IgG as measured during stress (Swanson, 2002). The results did show that the strain of LAB they used from the commercial product was able to colonize the gut but that the probiotic had no
effect on the ability of the foal to cope based on the parameters of stress that they measured.

**Summary**

Routine horse management practices such as diet and transportation have been shown to create physiological and psychological stress in the horse. With an increase in cortisol production from stress, changes can occur in glucose metabolism and insulin secretion which can negatively affect their pathways so they do not function efficiently. Increased cortisol has also been shown to cause changes in the microflora of the horse and impair immune responses. The goal of the present studies is to evaluate the effects of dietary manipulation, through feeding frequency, meal order or probiotic supplementation, on physiological and psychological responses of Quarter Horses in varying stages of production.
CHAPTER 2

Effects of Feeding Practice on Glucose, Insulin, Cortisol and Behavioral Responses
in Quarter Horse Mares
ABSTRACT

Today’s horse management practices often include restricted access to forage and feeding large quantities of grains in a limited number of meals throughout the day. These practices may create psychological and physiological stress in the horse, leading to increased cortisol concentrations which can inhibit the action of insulin and increase blood glucose levels. In a 6 x 6 Latin Square design, horses were fed identical diets consisting of 0.5% BW of a 12% CP pellet and 2% BW of mixed grass hay. Diets differed in the frequency (1 meal/d, 2 meals/d, or 3 meals/d) and order in which hay and grain were fed. Blood samples to determine glucose, insulin, and cortisol concentrations were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, 12 h following the morning meal on d 7 of each period. Behaviors were observed each day before and after meal times (07:30-9:00, 12:30-14:00, and 17:30-19:00) using a scan sampling technique (6 scans/horse/5 min for 1.5 hr). Instantaneous scans (1 scan/horse/min for 2 h) were used to observe and record behaviors during daily turnout (9:30-11:30). Analysis of variance for repeated measures showed that glucose, insulin, and cortisol concentrations varied with time; however, there were no differences due to feeding frequency or order. No differences were observed in crib-biting or pawing; however, horses fed 1 meal/d with grain given before hay were observed weaving more often than horses fed 2-3 meals/d (P < 0.05).
INTRODUCTION

Horses evolved to eat small, frequent meals. However, domestic horses are often maintained in conditions very different from which they evolved (MacFadden, 1995). Typical management practices in the United States often include restricted access to forage and feeding large quantities of concentrate in a limited number of meals throughout the day. Feeding less often reduces the cephalic response of digestion, increasing the need for insulin at an increased rate to transport glucose out of the blood and into cells (Power and Schulkin, 2008). These practices disrupt homeostasis in the horse, affecting glucose, insulin, and cortisol production (Youket et al., 1985). In addition, infrequent meals may lead to increases in stereotypic behaviors such as weaving and crib-biting (Cooper et al., 2005).

Glucose and insulin follow a comparable rhythm that is both due to a diurnal effect as well as the ingestion of meals. Glucose and insulin concentrations peak in the morning, but decrease as the day progresses (Stull and Rodiek, 1987). In addition, glucose and insulin increase after a meal is ingested, leveling out as glucose is transported by insulin into cells. Previous research shows that the normal pattern glucose and insulin exhibit when food is ingested may be affected by meal frequency (Youket et al., 1985; Stull and Rodiek, 1987). Ponies fed once a day had higher glucose concentrations and therefore increased insulin secretion in comparison to ponies fed more often (Youket et al., 1985). However, other studies did not find that glucose and insulin
were affected by meal frequency but rather glucose was changed due to diurnal effects and sampling time (Van Weyenberg et al., 2007). Increased cortisol concentrations, which also occur in Cushing’s disease, inhibit the action of insulin, resulting in increased blood glucose concentrations (Haffner et al., 2009).

The effects of the order of feeding concentrate and hay have not been extensively researched. One study observed differences between horse preference for hay and grain, measured by the number of times that a horse pressed a panel to receive the next portion of a meal (Elia et al., 2010). Horses that were fed grain first pressed the panel less often than the horses that were fed hay first. Although these results do not directly correlate to increased cortisol production, there may be increased stress with anticipation (Power and Shulkin, 2008), and it can be noted that horses anticipated grain more than hay. The results of this study are not universal. The order of diet has also been examined in terms of hematological values. The difference in starch digestion between alfalfa and oats in meal order has been researched in order to test for differences within the meal. In the study it was found that regardless of the meal order, there were no differences in pre-caecal starch digestion (Vervuert et al., 2008). Horses were put on an 11.5 h fast with a 10 d acclimation period before being given the diet. Although oats alone did cause an increase in glucose concentrations compared to oats fed with alfalfa, there were no differences within the diets when oats and alfalfa were fed together.

Glucose and insulin concentrations have been shown to change in regard to feeding order in exercise studies (Pagan and Harris, 1999). Feeding hay before grain in this particular study was associated with decreased glucose and insulin response as
measured through AUC. In contrast, horses that were fed hay after grain were measured to have higher glucose and insulin concentrations which did not decrease to match the other treatments until 4 h post grain. In the parameters measured, heart rate, indicative of stress, differed based on what portion of the meal was fed first. In the diet protocol of hay before grain, there was increased heart rate associated with post-exercise horses compared with the other diets. Horses that were given hay 4 h before grain had increased glucose and insulin concentrations, while horses fed a simultaneous meal of grain and hay had the normal trend of glucose and insulin when a meal is ingested (Pagan and Harris, 1999).

Feeding large quantities of feed not only causes gastrointestinal disruptions, but feeding infrequent meals may lead to increases in stereotypic behavior such as weaving and cribbing. Therefore to mimic the natural grazing pattern of the horse and decrease the incidence of stereotypic behavior, it may be beneficial to divide larger portions of the daily ration into smaller, more frequent meals.

Altering feeding management practices can influence physiological stress and alter metabolic function of hormones. However, because the implications are still unclear, the objective of this study was to evaluate the effects of feeding an identical diet, with different meal frequencies and order of delivery, on stereotypic behaviors and glucose, insulin and cortisol concentrations in horses.
MATERIALS AND METHODS

Experimental Design

Six Quarter Horse mares (7 ± 5 yr; 524 ± 87 kg) were used in a 6 x 6 Latin square design. Each horse received a similar quantity of feed per day (2.0% of BW of a mixed-grass hay and 0.5% BW of a 12% CP pelleted concentrate) and was randomly assigned to one of six feeding protocols during each 7 d period: 1 meal/d with grain fed first followed by hay 15 min later (1GH) or hay fed first followed by grain 15 min later (1HG), 2 meals/d with grain fed first followed by hay 15 min later (2GH) or hay fed first followed by grain 15 min later (2HG), 3 meals/d with grain fed first followed by hay 15 min later (3GH) or hay fed first followed by grain 15 min later (3HG). Throughout the study, the horses were housed and fed individually in 3.0 x 3.4 m stalls facing each other and had access to water and a loose trace mineral salt ad libitum. Horses were given free access to exercise for 2 hours/d in a 30.5 x 60.9 m sand arena. Behaviors were observed each day before and after meal times (07:30-9:00, 12:30-14:00, and 17:30-19:00) using a scan sampling technique (6 scans/horse/5 min for 1.5 hr). Instantaneous scans (1 scan/horse/min for 2 h) were used to observe and record behaviors during daily turnout (9:30-11:30). On a weekly basis, horses were weighed, body condition score (BCS) assessed (Henneke, 1983) and fecal samples were taken to evaluate changes in pH.

On d 6 of each period, horses were catheterized in the jugular vein following completion of the afternoon meal. The catheters were flushed with heparin in isotonic saline (10U of heparin/mL of saline) that evening and again in the morning prior to the start of the blood collections. On d 7 of each period, blood samples for determination of
plasma glucose (mg/dL), serum insulin (IRI; µIU/mL), and plasma cortisol (µg/mL) were taken 30 min before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, 12 h following the morning meal.

**Blood Sample Analysis**

Blood was allowed to clot for 10-15 min after collection and then centrifuged at 2500 x g. Serum was stored in vacutainer tubes at -80°C until further analysis. Blood samples for glucose analysis were collected into a vacutainer that contained a clotting agent to store serum, while insulin was collected into a vacutainer with fluoride and oxalate to prevent activate enzyme function. Plasma concentrations of glucose were determined using an YSI 2700 Biochemistry Analyzer (2300 STAT Plus; YSI, Yellow Springs, OH). Cortisol and insulin concentrations in serum were evaluated using a commercially available radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA). The kit was previously validated for specificity and accuracy in equine serum (Reimers, 1981).

**Statistical Analysis**

Data were analyzed using a mixed model with the MIXED procedure of SAS v. 9.2 (SAS Institute Inc, Cary, North Carolina) with frequency, order, and time and all 2 way and 3 way interactions of fixed effects and horse and period as random factors. The correlation of errors was modeled using the REPEATED statement. A variety of error
variance-covariance matrices were investigated; the first-order heterogeneous structure (ARH(1)) was deemed best based on Bayesian Information Criterion (BIC).

\[
\text{Glucose}_{ijk} = \mu + P_i + H_j + F_k + O_l + FO_{kl} + e_{ijkl}
\]

\[
\text{Insulin}_{ijk} = \mu + P_i + H_j + F_k + O_l + FO_{kl} + e_{ijkl}
\]

\[
\text{Cortisol}_{ijk} = \mu + P_i + H_j + F_k + O_l + FO_{kl} + e_{ijkl}
\]

Where P = period, H = horse, F = frequency, O = order

**RESULTS**

All horses consumed all of the food offered throughout the study. Body weight and fecal pH were not affected by feeding practice (frequency or order) during the experiment.

Basal plasma glucose concentrations immediately prior to feeding the morning meal ranged from 0.53 mg/dL to 0.99 mg/dL. Glucose levels were significant over time across all diets (P < 0.0001) with higher concentrations in the morning and lower peaks later in the day. However, no differences in plasma glucose were observed due to feeding frequency or meal order. Horses fed 1 meal/d tended to have higher plasma glucose levels for 4 h following the morning meal compared to horses fed 2 or 3 meals/d. Plasma glucose responses measured by area under the curve (AUC) were similar regardless of feeding practice (Table 1) due to the large amount of variation between individuals (Table 2). In addition there was considerable variation in the time of peak glucose concentration (Table 3).
Serum IRI concentrations immediately prior to feeding the morning meal ranged from 0.010 µIU/mL to 24.89 µIU/mL and peaked with all six feeding protocols within 1.5 to 4.5 h after the morning meal. Insulin levels were significant over time (p < 0.0001) and followed a similar pattern of glucose with higher concentrations in the morning that decreased throughout the day (Fig. 2). Feeding frequency or meal order did not have an effect on insulin concentrations. Insulin responses measured through AUC were similar regardless of feeding practice (Table 1).

Plasma cortisol concentration ranged from 1.95 µg/dL to 12.33 µg/dL immediately prior to the morning meal with peak concentrations of 2.90 µg/dL to 15.30 µg/dL. Plasma cortisol concentrations varied with time (p < 0.0001) but were not affected by feeding frequency or meal order (Fig. 3). Cortisol concentrations also increased following the afternoon and evening meals but not to the levels observed following the morning meal.

No differences were observed in crib-biting, or pawing, however, horses fed 1 meal/d (GH) were observed weaving more often than horses fed 2-3 meals/d (GH and HG; P < 0.05) (Fig. 4).

**DISCUSSION**

Current horse management practices often include feeding horses two large meals per day with concentrates often fed immediately prior to hay. This practice can increase stress in the horse from boredom or anticipation of the next meal, as well as change blood glucose and insulin levels (Youket et al., 1987; Cooper et al., 2005; Power and Shulkin,
2008). In the present study, blood glucose and insulin concentrations were not found to be manipulated by feeding frequency or the order in which hay and grain were fed. This could be due to the degree of variation that existed between individual horses response to diets, the sampling times, or seasonal changes.

Although there were no differences in insulin concentrations due to feeding frequency or order, there was a large increase in the insulin level of horses fed 3HG at 4 h after the morning meal compared to 3GH and horses fed 2 meals/d. The reason for this peak is not known but may be due to anticipation of the afternoon meal which could increase insulin secretion through a more dynamic cephalic response (Power and Shulkin, 2008). These observations can also be correlated with feed preference that horses have for grain to hay (Elia et al., 2010). Because the horses learned that grain would come after hay after the adaptation period, there could have been increased anticipation to receive the grain. Increased motivation and anticipation, to a certain extent, can lead to an extended cephalic response to produce more insulin (Power and Shulkin, 2008). A direct result of more insulin produced prior to a meal, allows for glucose clearance faster.

The most probable reason for the large insulin peak is individual variation. As shown through individual horses’ response to glucose and insulin over time, there was a lot of variation between the response of insulin and glucose to a diet. Not all horses responded the same way in which lower concentrations of glucose and insulin were associated with being fed more frequently.

Cortisol secretion follows a circadian rhythm in the horse, with peak secretion occurring in the early morning hours (Irvine et al., 1994). Previous research has
demonstrated that this rhythm may be disturbed by exercise, excitement, and a variety of other stressors (Irvine et al., 1994). However, in the present study, meal frequency and the order in which grain and hay were delivered did not affect cortisol concentrations.

In conclusion, this study suggests that meal frequency and order did not influence glucose and insulin concentrations in healthy horses. Although cortisol does not seem to be altered through frequency and order, smaller and more frequent meals may be beneficial for horses with metabolic disorders in which low blood glucose levels must be maintained.
CHAPTER 3

Effects of Probiotic Supplementation on Stress and Immune Responses in Young Quarter Horses
ABSTRACT

Today’s horse management practices often include restricted access to forage and feeding large quantities of grain in a limited number of meals throughout the day. These practices may create physiological and psychological stress in the horse. Increased stress produces cortisol which in turn can affect the microflora and immune responses in the horse. Twelve Quarter Horses were randomly assigned to one of two treatment groups: Control or Probiotic. Horses receiving probiotics were fed a commercial probiotic containing *E. faecium, L. acidophilus, L. casei*, and *L. plantarum* throughout the study. Fecal samples were collected weekly for microbial analysis. After 45 d, horses were vaccinated against tetanus toxoid. Blood samples were taken at the beginning of the experiment and on d 0, 7, 14, 21 and 28 d post-vaccination to determine tetanus antibody titers. On d 28 post-vaccination, horses were transported for 15 min and blood samples to determine cortisol concentrations were taken by jugular venipuncture immediately before and after transport. DGGE analysis revealed no differences in the microbial profiles of the horses due to probiotic supplementation. However, the microbial profiles of horses of comparable age showed similarities in banding patterns. Tetanus antibody titers increased in response to vaccination in all horses, regardless of probiotic supplementation. Similarly, there were no differences in plasma cortisol concentrations due to supplementation with probiotics. Plasma cortisol levels increased significantly
after transport within the control group (P < 0.05). In this study, supplementing a horse’s diet with probiotics did not influence stress and immune responses in young horses.

**INTRODUCTION**

Today’s horse management practices often include restricted access to forage and feeding large quantities of concentrates in a limited number of meals throughout the day. These practices may create physiological and psychological stress in the horse, leading to increased cortisol production which, in turn, may affect numerous metabolic pathways and the immune system (Blum et al., 2002). Stressors such as change in diet, transport, and weaning can lead to changes in the composition, diversity, and number of microorganisms residing in the gut, impairing both the horse’s performance and overall health (Bailey et al., 2011). Because many stress events are unavoidable, it is important to find ways to minimize any negative effects associated with stress. Probiotics may be one such strategy to combat problems associated with stress.

Probiotics are live microorganisms that can be given orally to influence the intestinal microflora to benefit the host (Teitelbaum and Walker, 2002). An effective probiotic is resistant to gastric acid and pancreatic secretions, has the ability to colonize the gastrointestinal tract, and inhibits the growth of pathogens. Because probiotics have been shown to have positive health effects on humans, the use of probiotics has extended to production animals due to the interest and need for alternatives to antibiotics (Smidt et al., 2011). Probiotics have also been utilized during times of stress for the horse (Weese and Rosseau, 2005). Cortisol production from stress functions as an immunosuppressant
to down-regulate the innate immune system (Bailey et al., 2011). Probiotics are believed to reconstitute the balance of microflora in the gut and influence the immune response through cytokine production (Blum et al., 2002). Although the mode of action varies based on the strain of bacteria, the health of the host, and the existing bacteria, it is generally believed that through competition for nutrients and binding sites, probiotics can limit pathogenesis (Blum et al., 2002).

The use of probiotics against pathogenesis has been examined with mixed results. Ward et al. (2004) found that at-risk horses, compromised due to colic surgery or antibiotics, could be treated with probiotics to inhibit the colonization of Salmonella. Although the probiotics reduced the incidence of Salmonella there were questions regarding the correct dosage and optimum time for treatment (before or after stress). Swanson (2002) reported that foals given a probiotic had higher lactate and lower acetate concentrations than control horses. However, there were no variables measured that related to a change in how the foal coped during weaning in terms of behavior and immune function. In another study, probiotic supplementation was shown to further harm foals that had diarrhea (Weese and Rosseau, 2005). After being supplemented with L. pentosus, there was a further decline of health associated with an abnormal clinical exam when foals were supplemented in comparison to control horses.

Although several studies have looked at the effect of probiotics on gastrointestinal microflora, their effect on immune function has not been studied fully in horses due to the strain specificity to the gut environment, health of the host, as well as mode of action (Blum et al., 2002). In humans, probiotics are able to regulate the immune system based
on the cytokines produced (Blum et al., 2002). By producing IL-2, probiotics can enhance cellular function of the immune system by increasing macrophage production of nitric oxide (Isolauri et al., 2001). In contrast, probiotics have also been shown to increase antibody production through IgA (Blum et al., 2002). With an increase in gut associated IgA, there is decreased permeability of the brush border to pathogenesis. IgA is also associated with creating an anti-inflammatory state in the gut (Blum et al., 2002).

According to product literature, mares supplemented with Probios had a 97% improvement in colostral IgG than mares in the control group (Freedom Health, Aurora, OH). This may be possible since probiotics can stimulate cytokine production to IL-10 to enhance antibody production; however this finding has not been replicated in other studies (Weese and Rosseau, 2005).

Other studies have claimed that probiotics have had no effects or harmful effects in horses (Weese and Rosseau, 2005; Parraga et al., 2008). This could be due to the immunocompetent state of the horses or the interaction of the specific strain used with the horses’ gut system. Established guts are more difficult to colonize due to competition for nutrients or if a horse is in a homeostatic state in both the outside environment and health (Weese and Rosseau, 2005). Therefore the objective of this study was to evaluate the effect of probiotic supplementation on immune response in healthy horses.

**MATERIALS AND METHODS**

Twelve Quarter Horses (1.6 ± 0.6 yrs) were used to evaluate the effects of probiotic supplementation on stress and immune response to tetanus toxoid vaccination.
The horses were randomly assigned to one of two treatment groups (probiotic or control). All horses received 0.5% BW of a 14% CP pelleted concentrate, with water and mixed grass hay *ad libitum* and were housed according to age in two outdoor paddocks, with access to shelter at all times. Horses in the probiotic treatment group were fed a commercial product containing *L. acidophilus, L. casei, L. plantarum,* and *E. Faecium,* according to the manufacturer’s recommendation. After 45 d, horses were vaccinated against tetanus toxoid (Super-Tet with Havlogen; Intervet Inc., Millsboro, DE). Blood samples were collected via jugular venipuncture for the determination of tetanus specific antibody titers on the first day of supplementation, immediately prior to vaccination (d 0) and on d 7, 14, 21, and 28 post-vaccination. To evaluate the effects of probiotic supplementation on stress responses as measured by cortisol production, horses were transported in groups of four, with two horses from each treatment group, separated by a divider, for 15 min. Prior to this study, only two horses had previous transport experience; all other horses were naïve to transport. On d 28 post-vaccination, blood samples to determine cortisol concentrations were taken by jugular venipuncture immediately before and after transport. Throughout the study, fresh fecal samples were collected weekly to measure pH and to evaluate changes in gut microflora. In addition, horses were weighed and BCS determined weekly (Henneke, 1983).

**Blood Analysis**

Blood was allowed to clot for 10-15 min after collection and then centrifuged at 2500 x g. Serum was stored at -80 °C until further analysis. Serum cortisol
concentrations in serum were evaluated using a commercially available radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA). The kit was previously validated for specificity and accuracy in equine serum (Reimers et al., 1981). Tetanus specific antibody titers (IE) were determined using an immunochromatography rapid test (TetaCheck; Fassisi, Munich, Germany).

**Microbial Analysis of Fecal Samples**

Fecal samples were stored at -80°C until further analysis. Microflora assessment was determined via Repeated Bead Beating Plus Column RB++C (Yu and Morrison, 2004) with a modified protocol for elution of adding 50 µL instead of 200 µL of AE. DNA extraction and purification was done using a Qiagen mini DNA kit (Qiagen Inc., Valencia, CA). Purified DNA was run on a 0.8% agarose gel at 100v for 1h. Quantification of DNA was determined via a nanodrop (NanoDrop Technologies, Montchanin, DE). DGGE-PCR was analyzed on DNA purified samples (Yu and Morrison, 2004). The primers used to derive 16S rDNA-trageted primers for amplification of all bacterial species were HDA1 (5’-3’): AC TCC TAC GGG AGG CAG CAG and HDA2 (5’-3’): GTA TTA CCG CGG CTG CTG CGA (Walter et al., 2000). A 40 bp GC clamp was attached to the reverse primer Lac2 to obtain PCR product suitable for DGGE. A 39 bp GC clamp was attached to the forward universal primer. The melting and annealing temperature for specific primers were determined and validated by Yu and Morrison (2004). The reaction mixture (50 µL) contained 0.255 µL
of each 100 uM primer and Taq polymerase, 1 µL of the DNA template, 1.02 µL of BSA, and 3.57 µL of 50 mM MgCl₂ and 0.408 µL dNTP.

**DGGE Protocol**

Prior to DGGE, 5 µL of each PCR product were subjected to 2% agarose gel electrophoresis to confirm successful amplification of the V3 region. Then, 10 µL aliquots of PCR product were resolved in a 7.5% polyacrylamide gel containing a 40%-60% gradient of denaturants (formamide and urea). The DGGE gel was run in 1% TAE at 60° C and 82 V for 16 h using INGENY phorU-2 (Ingeny; Leiden, The Netherlands) and the images were captured using a FluorChem® Imager (Alpha Innotech, San Leandro CA). Images were then analyzed with a software program to compare bands across gels (Bionumerics; Applied Maths, Austin, TX).

**Statistical Analysis**

Data were analyzed using a mixed model ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Institute Inc, Cary, North Carolina). Treatment and time were analyzed as fixed effects with horse and week as random factors. The correlation of errors was modeled using the REPEATED statement. A variety of error variance-covariance matrices were investigated; the first-order heterogeneous structure (ARH(1) was deemed best based on Bayesian Information Criterion (BIC).

\[
\text{Tetanus titers}_{ijk} = \mu + T_i + H_j + W_k + TD_{jk} + e_{ijk}
\]
Cortisol$_{ijk} = \mu + B_i + H_j + W_k + TB_{jk} + e_{ijk}$

Where D = treatment, H = horse, W = week, T=titers, B = time

**RESULTS**

All horses consumed all the food that was offered each day including the oral probiotic. Body weight and fecal pH were not affected by supplementation of the probiotic (data not shown).

Prior to vaccination, horses had little (0.5 IE/ml) to no (0 IE/ml) protection against tetanus. By d 7 post-vaccination, all of the horses in the study were fully protected (1.0 IE/ml) against tetanus, regardless of treatment (Fig.5).

Prior to transport, plasma cortisol concentrations ranged from 3.40 $\mu$g/dL to 6.35 $\mu$g/dL in the control group and 3.16 $\mu$g/dL to 7.17 $\mu$g/dL in the probiotic group. After 15 min of transport, plasma cortisol concentrations ranged from 4.37 $\mu$g/dL to 8.63 $\mu$g/dL in the control group and 4.66 $\mu$g/dL to 8.40 $\mu$g/dL in the probiotic group. Although no differences in cortisol production were found across treatment groups, control horses had significantly increased cortisol concentrations after transport compared to horses in the probiotic treatment group ($P < 0.05$) (Fig. 6).

PCR using universal primers to target all species in the V3 region of 16S rDNA from bacterial species was successful in amplifying the 200 bp region of interest in all fecal samples (Fig. 7). DGGE analysis revealed that the 2-year-old horses in the study
had a more diverse gut microflora compared to the yearlings regardless of probiotic supplementation and that it more closely resembled the profile of the probiotic (Fig 8.)

**DISCUSSION**

Current horse management practices can cause changes not only in the hormonal balances in the horse, but the makeup of gut microflora and immune responses (Buyse et al., 2006). When microbes are subject to increased cortisol concentration, there can be a decrease in the quantity, diversity, and ability of microbes to mount an immune response. To maintain a beneficial composition of microflora, probiotics have been introduced as a supplement with the meal. Through the enhancement of specific bacteria types, like *Lactobacillus*, that have been shown to assist against pathogens, there is a boost in host performance (Haghihi et al., 2006). Although the mode of action is not specifically known and clear, LAB type species have been shown to, once colonized, enhance immune responses through binding to phagocytes which can go on to secrete cytokines to enhance the overall immune response (Gourbeyre et al., 2008). The ability of probiotics to first colonize the gut and then enhance immune response appears to be species specific, status specific, as well as environment specific (Weese, 2002). In the current study, 12 healthy, young Quarter Horses, were not affected by probiotic supplementation.

Amplification of the V3 region was successful through universal primers HDA1 and HDA2 with a GC clamp. These results indicate that the DNA quality and quantity was sufficient enough to perform PCR and that there were viable bacterial species present in the probiotic product to which the primers could bind. Due to this, DGGE was utilized
for further analysis. The older horses had more common with the commercial probiotic then the yearlings as shown by band similarities. The most likely reason for this is due to the increased environmental exposure of the older horses compared to the yearlings. Another possible reason may be dose response. The 2 year-old horses used in the study weighed more than the yearlings and therefore received a greater dose of the probiotic.

Cortisol concentration is very strongly linked to stress events and a rise in cortisol production after transport stress has been extensively reported in horses (Bradshaw et al., 1996; Fazio and Ferlazzo, 2003; Fazio et al., 2008; Blandino et al., 2008). Although there were no differences in cortisol concentrations due to probiotic supplementation in the present study, there were differences within treatment groups. This result is similar to Weese and Rosseau, 2005 who found that there were no changes in diarrhea incidences, which would be a form of stress in mares supplemented with a probiotic in comparison to a control group of mares.

In conclusion, this study suggests that gut microflora diversity, cortisol response to transport stress, and immune responses in young horses were not affected by supplementation of a commercial probiotic product.
REFERENCES


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APPENDIX: TABLES AND FIGURES

60
Table 1. Effect of Feeding Frequency and Order on Glucose and Insulin AUC Concentrations in Quarter Horse Mares

<table>
<thead>
<tr>
<th>Diet</th>
<th>Glucose AUC (mg/dL)</th>
<th>Insulin AUC (µIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1GH</td>
<td>10.15 ± 0.11</td>
<td>213 ± 13.27</td>
</tr>
<tr>
<td>1HG</td>
<td>10.76 ± 0.16</td>
<td>302.79 ± 16.12</td>
</tr>
<tr>
<td>2GH</td>
<td>9.91 ± 0.07</td>
<td>239.65 ± 12.42</td>
</tr>
<tr>
<td>2HG</td>
<td>9.54 ± 0.05</td>
<td>239.79 ± 8.57</td>
</tr>
<tr>
<td>3GH</td>
<td>9.80 ± 0.06</td>
<td>330.81 ± 22.10</td>
</tr>
<tr>
<td>3HG</td>
<td>9.73 ± 0.06</td>
<td>238.12 ± 16.51</td>
</tr>
</tbody>
</table>

Pre- and post-prandial AUC glucose and insulin concentrations in Quarter Horse mares fed identical diets that differed in meal frequency and order. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11,11.5,and 12 h following the morning meal on d 7 of each period. AUC calculations are based on the trapezoidal rule (Soma et al., 2004). Data are presented as mean ± standard deviation.
Table 2. Effect of Feeding Frequency and Order on Glucose and Insulin AUC Concentrations in Individual Quarter Horse Mares on Diets.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Diet</th>
<th>Glucose AUC (mg/dL)</th>
<th>Insulin AUC (µIU/mL)</th>
<th>Horse</th>
<th>Diet</th>
<th>Glucose AUC (mg/dL)</th>
<th>Insulin AUC (µIU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1HG</td>
<td>11.17 ± 0.19</td>
<td>336.26 ± 13.53</td>
<td>1</td>
<td>1GH</td>
<td>11.90 ± 0.09</td>
<td>492.98 ± 30.17</td>
</tr>
<tr>
<td>2</td>
<td>1HG</td>
<td>9.78 ± 0.23</td>
<td>247.96 ± 16.17</td>
<td>2</td>
<td>1GH</td>
<td>9.41 ± 0.21</td>
<td>234.43 ± 37.36</td>
</tr>
<tr>
<td>3</td>
<td>1HG</td>
<td>12.96 ± 0.33</td>
<td>135.70 ± 6.36</td>
<td>3</td>
<td>1GH</td>
<td>11.16 ± 0.27</td>
<td>112.64 ± 3.89</td>
</tr>
<tr>
<td>4</td>
<td>1HG</td>
<td>10.73 ± 0.11</td>
<td>98.81 ± 7.52</td>
<td>4</td>
<td>1GH</td>
<td>10.80 ± 0.13</td>
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Pre- and post-prandial AUC glucose and insulin concentrations in individual Quarter Horse mares fed identical diets that differed in meal frequency and order. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, and 12 h following the morning meal on d 7 of each period. AUC calculations are based on the trapezoidal rule (Soma et al., 2004). Data are presented as mean ± standard deviation.
Table 3. Individual Time to Peak Glucose, Insulin, and Cortisol Concentrations in Response to Diets Varying in Frequency and Order

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<th>Horse</th>
<th>Diet</th>
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<th>Peak Insulin (hr)</th>
<th>Peak Cortisol (hr)</th>
<th>Diet</th>
<th>Peak Glucose (hr)</th>
<th>Peak Insulin (hr)</th>
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Glucose, insulin, and cortisol concentrations for individual Quarter Horse mares fed identical diets that differed in meal frequency and order at the time of the peak concentration. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, and 12 h following the morning meal on d 7 of each period. Data are presented as mean ± standard deviation.
Figure 1. Effect of feeding frequency and order on plasma glucose concentrations in Quarter Horse mares. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, and 12 h following the morning meal on d 7 of each period. Plasma glucose concentrations were significant over time (P < 0.05) but were not affected by feeding frequency or meal order.
Figure 2. Effect of feeding frequency and order on serum insulin concentrations in Quarter Horse mares. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, and 12 h following the morning meal on d 7 of each period. Serum IRI concentrations were significant over time (P < 0.05) but were not affected by feeding frequency or meal order.
Figure 3. Effect of feeding frequency and order on plasma cortisol concentrations in Quarter Horse mares. Blood samples were taken via jugular catheter 0.5 h before the morning meal and at 0, 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5, and 12 h following the morning meal on d 7 of each period. Plasma cortisol concentrations were significant over time (P < 0.05) but were not affected by feeding frequency or meal order.
Figure 4. Effect of feeding frequency and order on incidence of pawing, weaving, and crib-biting in stall of Quarter Horse mares. Behaviors were observed each day before and after meal times (07:30-9:00, 12:30-14:00, and 17:30-19:00) using a scan sampling technique (6 scans/horse/5 min for 1.5 hr). No differences were observed in crib-biting or pawing; however, horses fed 1 meal/d (GH) were observed weaving more often than horses fed 2-3 meals/d (GH and HG; P < 0.05).
Figure 5. Effect of probiotic supplementation on tetanus antibody titers in Quarter Horses. Blood samples were collected via jugular venipuncture for the determination of tetanus specific antibody titers on the first day of probiotic supplementation (d-45), immediately prior to vaccination (d 0) and on d 7, 14, 21, and 28 post-vaccination.
Figure 6. Effect of probiotic supplementation on cortisol concentrations pre- and post-transport in Quarter Horses. Horses were transported in groups for 15 min. Blood samples were collected via jugular venipuncture immediately before and after transport. Although no differences in cortisol production were found across treatment groups, control horses had significantly increased cortisol concentrations after transport for 15 min (P < 0.05).
Figure 7. Effect of probiotic supplementation on microbial profiles in the gastrointestinal tract of Quarter Horses as shown through PCR. Figure 4A is the microbial profile of total bacteria in horse feces of a horse in the control group. Figure 4B is the microbial profile of total bacteria in horse feces of a horse in the probiotic group. Lane 1 represents the negative control. Lane 2 represents the probiotic used in this study. Lane 3 represents the sample taken at the beginning of the supplementation period. Lane 4 represents the sample taken on the day of vaccination (d0) against tetanus. Lane 5 represents the sample taken 7d post-vaccination. Lane 6 represents the sample taken 14d post-vaccination. Lane 7 represents the sample taken 21d post-vaccination. Lane 8 represents the sample taken 28d post-vaccination.
Fig 8. Effect of probiotic supplementation on microbial profiles in the gastrointestinal tract of Quarter Horses as shown through DGGE. Analysis on V3 region of gastrointestinal isolates with an 8% polyacrylamide denaturing gradient gel. Lane 1 is the 100 bp ladder for reference to HDA1GC and HDA2 amplification. Lane 2 is the commercial probiotic product. Samples were grouped together after DGGE analysis by age (Bionumerics; Applied Maths, Austin, TX).