## ABSTRACT

GLUCK, CASSANDRA ROXANNE. The Fecal Microbiome and In-Vitro Fermentation, Glucose and Iron Metabolism, Inflammatory Cytokines and Performance Analyses in Horses. (Under the direction of Dr. Shannon Pratt-Phillips).

The hindgut houses a microbial community, in which microbes produce volatile fatty acids (VFAs). Study 1 assessed the fecal microbiota and fermentation in three equine populations with different habitual diets. Fecal samples were taken from: the feral Shackleford horses (n=24) and two domesticated populations: 1) NCSU Equine Educational Unit horses (n=18) and 2) privately owned horses (n=36) and the 16S rRNA gene was sequenced for microbiome analysis. An *in-vitro* fermentation model was also performed to quantify gas and VFA production. There were clear differences in the microbial composition, in which Clostridiales were more enriched in feral horses while Erysipelotrichales and Bacteroidales were more enriched in the privately owned and NCSU population, respectively. Acetate was the highest produced VFA during the *in-vitro* fermentation. Results indicated microbiota differences and subsequent fermentation when comparing domesticated versus feral horses, likely driven by the habitual diet.

The next three studies focused on iron metabolism. The NRC recommends 500 kg horses at maintenance consume 400 mg iron daily, however, it has been reported that horses may consume much higher quantities. There has also been concern over iron intake and its possible correlation to insulin dysregulation (ID) and increased body iron stores. In Study 2, glucose and iron metabolism parameters were assessed in horses kept on mixed pastures (n=21). Insulin values were 19.5±10.00 µU/ml while 85% of ferritin levels (409.2±164.50 ng/ml) were above the reference range. Although Pearson's correlation found no correlation between ferritin and insulin when considering all horses, a separate Pearson's correlation revealed a strong, positive relationship in horses with insulin >20 µU/ml (indicative of potential hyperinsulinemia) and

ferritin. Results warrant further investigation into the relationship among dietary iron, insulin and ferritin.

Mixed-breed geldings (n=12) were used for Study 3 and 4. During the first 28 days (Hay Phase), all horses were fed mixed-grass hay and meeting NRC iron recommendations. At the end of this phase, an oral sugar test (OST), 24-hour fecal collection and hematology cell counts were performed. For the latter 28 days (Supplement Phase), horses were randomly assigned to continue on the hay diet (CTRL; n=4) or be supplemented with an iron supplement daily (IRON; n=8), in which IRON horses were consuming 10x the daily recommendations. Subsequent testing was done at the end of the Supplement Phase. Serum ferritin was higher in IRON horses across the 2 phases. Sixty-minute insulin was not indicative of ID (>65 μU/ml) in IRON horses, however insulin responses were higher compared to controls. Results indicate plausible correlations among increased iron intake, body iron status and insulin responses.

Study 4 utilized an iron absorption test (IAT) to assess iron status. Although IATs have been used in humans, IATs have not been previously done in horses. Two IATs were performed, one on day 5 of the Hay Phase (Pre) and another one at the end of the Supplement Phase (Post). Jugular venous samples were taken, an iron dose was given orally and then subsequent samples were taken at 3-, 6-, 12- and 24-hours. There was a phase x treatment interaction for ferritin while serum hepcidin responses were higher in IRON horses compared to controls. Results indicate that a large iron dose will increase ferritin and hepcidin responses, especially in supplemented horses. Thus, IATs may be useful to investigate iron status in horses.

Study 5 focused on TNF-alpha concentrations in elite competition ponies. Samples and data from a previous study were utilized to analyze TNF-alpha and compared for possible correlations. Ninety-one percent of ponies (n=23) were considered fleshy or obese. Pearson’s

correlation revealed a small, positive relationship between TNF-alpha and insulin. Various factors possibly affected TNF-alpha; however, it appears there are correlations between insulin and TNF-alpha in elite competition ponies.

Performance analysis is used in sports like soccer to provide feedback and enhance performance. However, this has not been implemented in equestrian sports. Study 6 utilized notational analysis to determine if course variables affected performance at a national pony competition. Show jumping rounds were watched online, fault(s) occurrences were recorded and statistical analysis assessed possible relationships. Faults were more likely to occur during the final quarter of the course compared to the first. Results indicate that performance analysis techniques may be useful to inform training and riding strategies.

# The Fecal Microbiome and In-Vitro Fermentation, Glucose and Iron Metabolism, Inflammatory Cytokines and Performance Analyses in Horses

by
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## DEDICATION

I dedicate this dissertation to everyone who supported me along this journey as I pursued my PhD.

## BIOGRAPHY

Cassandra Roxanne Gluck grew up in Ventura, California where her passion for horses started at a young age. Cassandra began taking riding lessons at two years old and after riding Western for a few years, she eventually switched to English and learned to jump. After graduating high school, Cassandra decided to attend the University of Massachusetts at Amherst where she was an Animal Science/Pre-Veterinary major. She was also a show team member on the Intercollegiate Horse Show Association (IHSA) equestrian team at UMass and enjoyed "catch riding" in equitation classes at IHSA shows. After 2 years at UMass, Cassandra decided she wanted to finish her degree closer to home and transferred to California Polytechnic State University, San Luis Obispo (Cal Poly).

After transferring to Cal Poly, Cassandra still intended to go to vet school. However, mid-way into her junior year, she realized she wanted to explore other career options and was interested in nutrition research. During her time at Cal Poly, Cassandra was an undergraduate research assistant for a digestibility trial under Dr. Mark Edwards and his Master's student, Emily Schwartz. During the digestibility trial, Cassandra helped with feeding the horses, collecting fecal samples with fecal collection harnesses and assisting with data collection. After being involved with this project, she realized she didn't want to pursue veterinary school and was instead interested in going to graduate school for equine nutrition. In order to gain more research and equine nutrition experience, Cassandra applied and was selected to participate in the summer internship program at Kentucky Equine Research (KER) in Versailles, Kentucky. During her internship at KER, she helped with nutrition and exercise physiology research going on at the farm, which included learning how to run horses on the equine treadmill. After the KER

internship, Cassandra finished her Bachelor's degree in Animal Science and Equine Science minor at Cal Poly.

Instead of going straight into graduate school, Cassandra took a few years off of school and was an outside sales representative in Northern California for a few equine feed companies, in which she learned how to talk to horse owners and trainers about nutrition and feeds in an easy-to-understand way. However, after a few years, Cassandra decided to apply to graduate school and was accepted to North Carolina State University to work with Dr. Shannon Pratt-Phillips. Cassandra (and her now husband Matt) moved from California to Raleigh in July 2020 for her to begin her graduate studies. Initially coming to NC State as a Master's student, Cassandra worked under Dr. Pratt-Phillips for a year before deciding to go straight into a PhD program. Cassandra has completed several projects related to equine nutrition throughout her time as a graduate student and has presented at both national and international scientific conferences. Additionally, she has developed a love for teaching by being a graduate teaching assistant for several laboratory courses.

Upon completion of her PhD in December 2024, Cassandra plans to continue teaching and pursuing research in equine nutrition. Ultimately, she wants to impact students' lives through education and continue to perform research to positively affect the health and welfare of horses.

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# CHAPTER 1: LITERATURE REVIEW

## PART I: MICROBIOME & IN-VITRO FERMENTATION

### 1.1. Introduction

The modern horse, known as *Equus caballus*, evolved about 1 million years ago from its ancestor *Eohippus*, an early equid ungulate (Goodwin, 2007). As horses evolved millions of years ago, this aligned with the development of grasslands. The environmental and geographic changes occurring during this period ultimately led to physiological and anatomical changes that enabled the horse to survive. Due to this, it is not exactly known whether the horse's gastrointestinal (GI) tract was modified to suit the grasses available or if the horse selected the type of food most suited for its GI tract (R. A. M. Al Jassim & F. M. Andrews, 2009). Horses are best classified as non-ruminant herbivores and hindgut fermenters due to their large and complex digestive system. The lower part of the digestive tract, also known as the hindgut, contains an enlarged cecum and colon, which houses an essential microbial community (Julliand & Grimm, 2016).

### 1.2. The Hindgut

The lower segment of the equine digestive tract, commonly known as the hindgut, consists of the cecum, colon (large and small) and rectum (Figure 1.1). A collective term for the colon (large and small) and rectum is the large intestine. This portion of the digestive tract comprises about 61% of the total volume of the digestive tract, with the cecum and large intestine being 16% and 45%, respectively. However, this portion is only 30% of the digestive tract length, the entirety of the tract being about 30 meters long (Ericsson et al., 2016; Geor et al., 2013). The large intestine (LI) is voluminous, holding about 95 to 112 liters of liquid, which is about the size of the stomach of an adult cow (R. A. M. Al Jassim & F. M. Andrews, 2009). The walls of the LI

contain circular and longitudinal muscle fibers in order to aid in digesta passage through this portion of the tract. Unlike other herbivores, the small intestine within the horse opens directly into the cecum through a muscular valve known as the ileocecal valve. Close to this valve is a second valve where digesta can pass from the cecum into the right ventral colon (R. A. M. Al Jassim & F. M. Andrews, 2009).

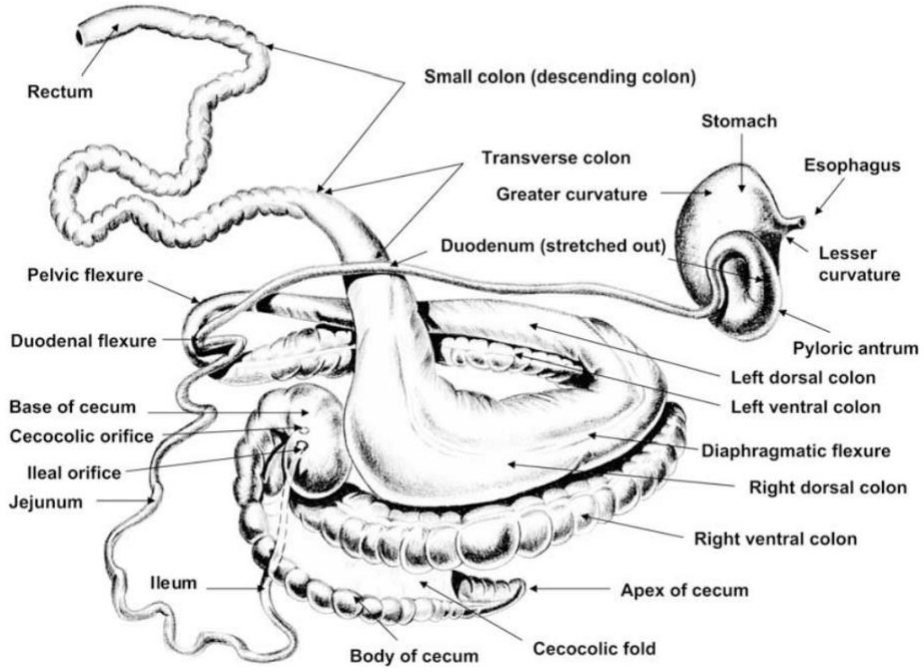


*Figure 1.1. Schematic of the equine digestive tract starting from the esophagus and continuing to the rectum. (Source: (R. A. Al Jassim & F. M. Andrews, 2009))*

The cecum is a large, mixing vat type structure extending from the pelvic inlet to the abdominal floor of the horse's right flank. Due to its large size and structure, it helps slow digesta passing through it, allowing the microbes to digest the fiber anywhere from 7 hours up to even days (Share et al., 2022). This structure lacks a longitudinal muscle layer and instead has muscle bands ("tarnia") that follow the length and gathers the cecum's wall into sacculations called "haustra". The haustra delay chyme transport by acting like buckets.

The ascending colon, known as the “large colon”, consists of four sections: right ventral, left ventral, right dorsal and left dorsal (Reece, 2005). The large colon is folded in order to form a double loop consisting of these four different sections, which are connected by the “sternal”, “pelvic” and “diaphragmatic” flexures. The transition segment between the ventral and dorsal colon, known as the pelvic flexure, is narrow at only 9 cm to delay the transport of large particles between these two regions, ultimately increasing fermentability and retention time. However, the decreased passage rate can allow heavy particles (like sand) to settle, which could result in an impaction or colic. Within the large colon, production of methane, ammonia, volatile fatty acids (VFAs), amino acids and B vitamins continues to occur through microbial fermentation (Share et al., 2022). Minerals and electrolytes (sodium, chloride and potassium) are also absorbed with water as ingesta material moves onto the small colon. The small colon is the final portion of the horse’s digestive tract and primarily absorbs water. Finally, fecal balls will be formed and move towards the rectum.

### 1.3. Role of the Equine Hindgut Microbiota

The hindgut of the horse primarily houses the microbial community. The relationship between the microbes and horse is mutualistic, in which the microbes consume substrates from the horse, therefore living in and reproducing in an ideal environment; whereas the horse receives by-products of fermentation of indigestible feed that can be metabolized to produce energy and the microbes synthesize B vitamins and Vitamin K, which are essential to the horse’s diet (NRC, 2007). The extent in which these vitamins are actually absorbed within the hindgut is unknown, however there is evidence that shows horses can survive without additional intake of these Vitamin K and the B vitamins (Geor et al., 2013). Additionally, microbes within the terminal small intestine will produce these vitamins in sufficient quantities and ultimately be

absorbed from the small intestine (Schenck & Kolb, 1982). Plant or fibrous materials not digested within the upper segment of the digestive tract are broken down and fermented by these microbes. A by-product of this fermentation are volatile fatty acids, also known as VFAs. The primary VFAs include acetate, propionate and butyrate, which provide about 60-70% of a horse's daily maintenance energy needs for idle horses (Argenzio & Hintz, 1972; Argenzio et al., 1974). This is similar to ruminants in which it is estimated that VFAs contribute about 70% of caloric requirements (Bergman, 1990). Due to the microbes' ability to break down and ferment these otherwise unusable materials, they serve an important role in order for a horse to utilize feed efficiency and ultimately survive by helping to meet energy needs.

Phyla commonly found within the equine hindgut include Firmicutes, Verrucomicrobia and Bacteroidetes (Costa et al., 2015; Dougal et al., 2013). When evaluating different compartments of the digestive tract, microbes are present in varying amounts as well as species based on the location within the GI tract. A study by Costa et al. found that when grouping and comparing the proximal part of the tract (stomach, duodenum and ileum) to the distal part (cecum, large colon, small colon, rectum and feces), the bacterial diversity within the proximal part was significantly lower than the distal part of the tract, indicating the hindgut hosts a more diverse microbial community (Costa et al., 2015). In a study assessing four sites of the hindgut, Reed et al. found that most taxonomic features identified in the study were generally shared by the cecum, ventral colon, dorsal colon and feces, and the closer the compartment was to the terminal end of the digestive tract, the more shared features there were. The study saw a large decrease in Bacteroidetes when comparing the cecum and ventral colon to the dorsal colon and fecal samples while Euryarchaeota increased after the pelvic flexure, which connects the left ventral and left dorsal colon (Lopes & Pfeiffer, 2000; Reed et al., 2021).

Collecting fecal samples is commonly used to evaluate the microbial community as this collection method is relatively easy to perform. However, these samples are generally regarded most representative of the tract beyond the pelvic flexure (Reed et al., 2021) so caution is needed when making generalizations about the microbial community within the proximal portions of the hindgut when utilizing fecal samples. This has been seen in various studies, one of which Dougal et al. did find differences in the microbial community between the cecal and right dorsal colon (Dougal et al., 2012; Reed et al., 2021). Studies utilizing fecal samples have found that Firmicutes is the most predominant (Costa et al., 2015; Costa et al., 2012; Dougal et al., 2013) phylum while either Verrucomicrobia (Costa et al., 2015; Shepherd et al., 2012; Steelman et al., 2012) or Bacteroidetes (Costa et al., 2012; Dougal et al., 2013) have been found to be the second most predominant within fecal samples.

Despite the research that has been done on determining the core microbial community, which is defined as the stable bacterial components including their key microorganisms and functions (Costa et al., 2015; Kauter et al., 2019), there is still more to learn about exactly which microbes make up this community within our horses. There is also still a lack of understanding on how a horse's diet and age, environmental and health issues such as laminitis, colic and others affect the microbial community in the short-term as well as long-term. Therefore, by gaining an understanding of the microbial community and how this can be affected by a plethora of factors, we can ensure the health of the equine population.

### 1.4. Diet and the Equine Microbiome

Many factors have been demonstrated to cause shifts in the equine microbiota, including diet, age, exercise, disease and stress (R. A. Al Jassim & F. M. Andrews, 2009; De La Torre et al., 2019; Ganda et al., 2023; Hansen et al., 2015; Milinovich et al., 2008; Proudman et al., 2015).

The effect of the diet has been studied within the equine microbiome field as it is easy to control and manipulate. Due to the importance the microbiota plays in utilizing dietary components not digested within the foregut, understanding the impact on how dietary management of horses as well as abrupt changes within the diet affect the microbiota is of the utmost importance. Studies have shown that health issues like colic and laminitis can result from dysbiosis within the hindgut due to dietary factors (Julliand & Grimm, 2017).

The composition of the diet, which is greatly influenced by the individual horse and overall management, is a determinant of the microbiota population. The University of Delaware Equine Microbiome Project obtained fecal samples from 184 horse owners who also filled out a survey questionnaire to determine dietary information. Horses primarily maintained on forage-concentrate diets had higher abundances of *Lactobacillus, Streptococcus* and RFN20 taxa while horses on pasture had higher abundances of *Prevotella, Oscillospira* and *Christensenellaceae* (Berg et al., 2017; Julliand & Grimm, 2017). These different abundances could be due to lactic acid bacteria counts reflecting a diet higher in starch (Milinovich et al., 2010), indicating the interconnectedness between the diet and microbiota composition. When controlling the composition of the diet to investigate how diets high in readily fermentable carbohydrates, which is traditionally used in performance horses to account for increased energy requirements, Willing et al. (2009) utilized a forage only diet high in energy as well as a traditional forage-concentrate diet to assess the effects on the microbiota. Horses fed the forage only diet had a more stable microbial composition and lower amounts of lactic acid bacteria, specifically members of the *Streptococcus bovis/equinus* complex, while horses on the forage-concentrate diet had motile and swarming *Lactobacillus ruminis*, which has not been previously reported in horses (Willing et al., 2009). Another study found that a proliferation of streptococcal species can result in laminitis

(Milinovich et al., 2008) and although Willing et al. (2009) did not see any incidences of laminitis, it was observed that horses fed the forage-concentrate diet had increased abundances of *Streptococcus bovis/equinus*. Lactic acid bacteria are also associated with gastrointestinal disorders, therefore Willing et al.'s study demonstrated that a forage-only diet may be suitable for prevention of these disorders (Willing et al., 2009).

Nutrient availability of the diet can also play a role in the microbial diversity and stability within the cecum. Cecal fluid was taken from horses fed either a low and slower nutrient availability diet in the form of hay or a high and rapid nutrient availability diet in the form of a mixture of hay and oats (Hansen et al., 2015). Horses fed the low nutrient availability diet (hay) had a higher level of diversity and stability when compared to horses fed the higher nutrient availability diet (oats). Additionally, horses fed the oats diet displayed overrepresentation of the bacterial family *Porphyromonadaceae* and high propionate concentrations within the cecum (Hansen et al., 2015). Members of this bacterial family have been shown to produce propionic acid upon metabolism of glucose (Shkoporov et al., 2013), indicating that dietary source plays a role in the microbes present and ultimately the VFAs produced within the hindgut. Horses fed the hay diet had both overrepresentation of Clostridiales and acetate production, however the role of this taxa is largely unknown and production of acetate is associated with a wide range of bacteria (Macfarlane & Macfarlane, 2011), so it is difficult to pinpoint which microbes typically produce this. In the study by Hansen et al. (2015), a positive relationship was observed between increased diversity and overall community stability, which is in accordance with ecological theory (Cooper et al., 2005; Hansen et al., 2015).

Oftentimes, horse owners will add supplemental feeds in order to add calories to meet increased energy requirements or to put weight on a horse. Calories are primarily added through

the use of fat and/or starch within equine diets. Typically, fat is added by top-dressing feed with some type of oil such as vegetable oil and starch can be added by utilizing feeds such as oats, corn and others. However, when these supplemental feeds are added, this could have an effect on the hindgut microbiota. Dougal et al. (2014) assessed the fecal microbiome of horses fed three different diets: hay only, hay with an oil rich supplement and hay with a starch rich supplement. When looking at the core community, core OTUs associated with the oil and starch rich diets were smaller (10.3% and 5.4% of sequences, respectively) compared to the hay only diet (15.9% of sequences). When considering both the diet and age of the horses within the study, the bacterial population was predominantly Firmicutes followed by Bacteroidetes. Furthermore, the only notable difference in relative abundance was seen with an increase in Proteobacteria in the oil rich and starch rich diets (Dougal et al., 2014), with previous work in cattle showing features (e.g., *Roseburia)* within the Proteobacteria phylum being higher in animals fed a high starch diet (Petri et al., 2013). Therefore, the microbial community will be influenced by composition of the diet within horses.

### 1.5. Disease and the Equine Microbiome

Several diseases have been shown to change the composition of the equine microbiota, including colic, laminitis, equine metabolic syndrome and others. Although it is important to assess the differences in the microbiota before, during and after a disease occurrence, it is difficult to assess this with sporadic diseases specifically. Furthermore, it is also difficult to differentiate cause versus effect (Costa & Weese, 2018). Due to the involvement of microbes in fermentation of digesta to produce VFAs within the hindgut, gastrointestinal disturbances can result in alteration of fermentation patterns, thus potentially leading to metabolic disorders (Dougal et al., 2013).

Colic is not a disease, rather it refers to abdominal pain that can be caused by several different problems and is the number-one cause of mortality in horses with 4.2 colic events per 100 horses occurring every year according to a survey from the National Animal Health Monitoring System. One point two percent of these colic events will be surgical while 11% will be fatal (Loving, 2016). Due to colic being caused by multiple risk factors, it is to be expected that changes in the microbiota will occur after colic, however the severity and cause may be different in each case (Costa & Weese, 2018). Although colic is very prevalent within our equine population, there are few studies assessing the consequences of colic on the microbiota. When collecting rectal samples from horses admitted for a non-surgical colic episode, Venable et al. found an increase in Bacteroidetes during a colic episode compared to post-colic samples taken (Venable et al., 2013). In another study evaluating the effects of post-partum colic in mares, a higher abundance of Proteobacteria was found in mares prior to colic compared to mares that did not colic. Furthermore, all samples with a relative abundance of ≤50% of Firmicutes preceded an episode of colic. Based on the findings, there is an association between Firmicutes and Proteobacteria and the onset of colic, indicating that the microbial composition of the hindgut could be used to predict and prevent colic episodes (Weese et al., 2015). When looking at horses with small intestinal colic or large intestinal colic (n=28) compared to healthy horses (n=24), Park et al. (2021) found that horses with intestinal diseases had less bacterial diversity compared to healthy horses. In addition, lactic acid bacteria were overgrown in horses with large intestinal colic while the Firmicutes: Bacteroidetes (F/B) ratio was 1,94, 2.37 and 1.74 in horses with large intestinal colic, small intestinal colic and healthy horses, respectively (Park et al., 2021). This study was in agreement with others in which the F/B ratio was increased in horses with intestinal conditions (Stewart et al., 2019; Weese et al., 2015) and in humans, this ratio has been indicative

of gut dysbiosis (Magne et al., 2020), however some other studies have reported increased Bacteroidetes in horses with colic (Stewart et al., 2019; Stewart et al., 2021). Therefore, the F/B ratio cannot be solely used to assess the status of intestinal diseases within horses.

Although colic can be caused by a number of issues, two areas that are associated with an increased risk are management changes and seasonal shifts. Management changes have been shown to cause shifts in the microbiota composition within the hindgut, which has led this to be a proposed underlying mechanism for a colic episode. However, there have been limited studies assessing the composition of the hindgut microbiota over a long-term period to see if and how the microbiota shifts in normal horses maintained at pasture with minimal management changes. When assessing the fecal microbiota over a 52-week period, Salem et al. (2018) found that Firmicutes and Bacteroidetes were predominant throughout the study. Additionally, supplementary forage, environmental conditions and season all played a significant role in shifts within the microbiota composition, indicating that the microbiota is highly dynamic (Salem et al., 2018). None of the study horses developed colic over the 52-week period, suggesting that the proposed correlation between management changes and colic risk from shifts in the microbiota is too simplistic, therefore needing to be potentially investigated further.

Laminitis is another common condition found within our equine population, constituting 7.5-15.7% of all lameness problems within the United States (USDA-NAHMS, 2000). Laminitis refers to inflammation and damage of the laminae tissue between the hoof and the coffin bone. This condition, like colic, can be caused by a number of factors, however one factor that has been well described in the onset of laminitis is carbohydrate overload from excess grain or pasture exposure (USDA-NAHMS, 2000). Since dietary changes can have effects on the microbial composition, this means that laminitis will also likely have an effect on the microbiota.

Oligofructose-induced laminitis was performed in a population of horses and cecal fluid was collected for 24 hours post-oligofructose administration. The equine hindgut streptococcal species *Streptococcus lutetiensis* was a predominant microorganism found prior to laminitis onset. *S. lutetiensis* is an oligofructose-utilizing microorganism, which produces large quantities of lactate (Milinovich et al., 2008). However, the lactate produced from the oligofructose fermentation may not be directly involved in the development of laminitis, indicating this is an area for further investigation.

Laminitis is a condition that can come and go throughout a horse's life or it can be a chronic condition, which means that if a horse has chronic laminitis, this may result in a permanent shift in the microbiota composition due to this. Steelman et al. (2012) evaluated the fecal microbiota between healthy control horses and horses with chronic laminitis. Horses with chronic laminitis had significantly higher (P=0.019) bacterial diversity when using operational taxonomic units (OTUs) and higher abundances of two undescribed Clostridiales genera (P=0.03 and P=0.01, respectively) when compared to the control horses. Interestingly, the genera *Streptococcus* was most abundant in 6 control horses and 3 horses with chronic laminitis, with variation of this genus being between 0.40-91.96% of all OTUs within healthy horses (Steelman et al., 2012). Based on individual variations in abundance of *Streptococcus* seen within healthy horses, this suggests this genus may not be closely linked with chronic laminitis, therefore differing to the link between this genus and the onset of oligofructose-induced laminitis seen in other studies (Garner et al., 1978; Milinovich et al., 2008).

Obesity is defined as an excessive accumulation of body fat which negatively impacts a horse's health (Rendle et al., 2018) and can lead to a number of health issues like insulin resistance, laminitis (Geor & Harris, 2009), metabolic syndrome and others. One practical tool

that can be used to assess if a horse may be obese is through the Henneke Body Condition Scoring (BCS) system, which measures fat deposition in 6 areas of a horse's body visually and via palpation (Henneke et al., 1983). The BCS system uses a 1-9 scoring system, in which Thatcher et al. (2008) identified a BCS <4 being considered under condition, a BCS 4-6 being ideal, a BCS 6.5-7 being over condition and a BCS 7.5-9 being obese (Thatcher et al., 2008). Obesity and its prevalence within our equine population has been assessed within the US, with Thatcher et al. (2008) finding 51% of horses being over condition and/or obese while Phillips et al. (2010) found 20% of horses were obese (BCS >7) and 28% were moderately fleshy (BCS 6-7) (Pratt-Phillips et al., 2010; Thatcher et al., 2008).

It has been suggested that Firmicutes and Bacteroidetes are involved in obesity development, with evidence showing that obese subjects displayed increased abundances of Firmicutes (Ley et al., 2006). When mice were fed a high-fat diet, microbial diversity was decreased while the Firmicutes: Bacteroidetes (F/B) ratio was increased (De Wit et al., 2012). Based on the results from these and other studies assessing obese animals and humans (Bäckhed et al., 2004; De Wit et al., 2012; Ley et al., 2005; Ley et al., 2006), it has been proposed that Firmicutes is more effective at extracting energy from food via fermentation versus Bacteroidetes, ultimately promoting weight gain (Magne et al., 2020). Thus, this ratio has been considered a potential hallmark for obesity (De Bandt et al., 2011; Zou et al., 2020). Although this ratio has been proposed as an indicator of obesity, there have been discrepancies in results. Schwiertz et al. (2010) assessed differences in the microbiota and fecal VFA concentrations in obese, overweight and lean humans (Schwiertz et al., 2010). The total VFA amounts were higher in the obese group compared to the lean group, with the highest increase being seen with propionate. When assessing this ratio, this changed in favor of Bacteroidetes within the obese and overweight

groups, thus indicating a difference in results compared to other studies (De Wit et al., 2012; Schwiertz et al., 2010). However, it is important to note that other compositional changes could be occurring at the family, genus or species level, which may be more relevant than the F/B ratio (Aguirre & Venema, 2015; Magne et al., 2020). The results of Schwiertz et al. (2010) show that in addition to the microbiota composition, VFA production and metabolism are likely playing a role within obesity.

As mentioned previously, obesity is prevalent within our equine population (Pratt-Phillips et al., 2010; Thatcher et al., 2008) so the need to assess its effect on the equine microbiome is important. Human studies have shown that microbial diversity is reduced in obese humans (Turnbaugh et al., 2009) but a study by Morrison et al. (2018) in Welsh Mountain ponies found that bacterial diversity increased in obese ponies (Morrison et al., 2018). Additionally, it was found that the relative abundances of Bacteroidetes, Firmicutes and Actinobacteria were greater in obese horses compared to controls. Despite human studies showing an association with obesity and the abundance of Bacteroidetes and Firmicutes, Morrison et al. (2018) did not see this association. Similarly, Biddle et al. (2018) identified an increase in bacterial diversity and abundance of Firmicutes but found a lower abundance of Bacteroidetes and Actinobacteria, however the differences seen in abundances of Bacteroidetes and Actinobacteria between these two studies could be due to differences in study design (Biddle et al., 2018; Morrison et al., 2018). Another study done by Morrison et al. (2020) induced weight loss in Welsh Mountain ponies through dietary restriction to evaluate the effects of this on the microbiome. Following weight loss, Firmicutes and Tenericutes were significantly reduced but Bacteroidetes did not change, which differs from human studies in which Bacteroidetes was increased following dietary restriction (Ley et al., 2006; Morrison et al., 2020).

Equine metabolic syndrome (EMS) is a collection of risk factors such as obesity and insulin dysregulation (Johnson et al., 2012), in which ponies and horses have a susceptibility to develop laminitis (Geor et al., 2013). As laminitis is a debilitating disease for horses, it is important to prevent this from occurring. Due to obesity being an issue within the equine industry and therefore EMS being an issue, it is important to understand the pathophysiology of this condition (Coleman et al., 2019). In humans, the microbiota within the digestive tract have been shown to play a role in the development of metabolic disease like type 2 diabetes by influencing energy metabolism (Musso et al., 2010) as well as gastrointestinal hormone secretion and inflammation (Bäckhed et al., 2007). However, the link between the microbiota and metabolic activities hasn't been as thoroughly explored in horses. In a study by Elzinga et al. (2016), horses with EMS (classified as having insulin dysregulation through an oral sugar test, general or regional adiposity and a history or predisposition to laminitis (Frank et al., 2010)), were compared to control horses to assess differences within the microbiota. This study found that the fecal microbiota of EMS horses was different in community structure and relative abundances. Additionally, horses with EMS possessed a decreased microbial diversity compared to control horses (Elzinga et al., 2016). However, EMS horses had higher abundances of a member of the subdivision 5 of the phylum Verrucomicrobia, which has been elucidated in obesity and metabolic disease and has been suggested as a biomarker for glucose intolerance (Zhang et al., 2013). Therefore, although this area needs to be explored further, it does appear that EMS plays a role within the composition of the microbial community.

### 1.6. Equine Microbiome in Domesticated versus Feral Populations

One area that can provide further insights on the impact of diet and management practices of domesticated horses on the equine microbiota is by comparing the similarities and or differences

of the microbiome in feral versus domesticated populations. There are a limited number of published studies within this area, likely due to the difficulty with obtaining samples from feral populations of horses. However, fecal samples can be collected relatively easily and analyzed in order to ensure there is no interference with a feral population.

In a study by Ang et al. (20220), fecal samples were collected from feral and domesticated horse populations from South America, Europe and Asia and analyzed using shotgun sequencing. Feral horses possessed a higher abundance of eukaryota and viruses. In addition, genes coding for enzymes involved in carbohydrate metabolism were significant higher across all feral horse populations. Within the domesticated horse populations microbiomes, genes enriched in tetracycline resistance were found, reflecting antibiotic use within these populations (Ang et al., 2022). Thus, it appears that management practices of domesticated populations influence the composition of the microbiota.

When evaluating the impacts of domestication and captivity by comparing the microbiomes of Przewalski's horse (PH; n=44) to domesticated horses (n=28) using 16S rRNA sequencing, the PH microbiome differed in composition and was more diverse compared to domesticated horses. The microbes of the two populations were distinct due to different ratios of taxa in the orders Clostridiales, Bacteroidales, Erysipelotrichales, and Spirochaetales. Interestingly, life history influenced the microbiome of the Przewalski's horses. The PH population born in captivity in European zoos (n=4) had a low diversity of microbiota compared to horses (n=35) born in natural reserves (Metcalf et al., 2017). These results showcase that the microbiome may be influenced from a young age by the mare's microbiota, although future work needs to be done in this area. Additionally, the associated management practices the mare is exposed to likely has an influence on their foal's microbiome as it continues to mature (Quercia et al., 2019).

The equine fecal microbiota may undergo compositional changes based on the level of domestication. In the United Kingdom, fecal samples were obtained from Exmoor ponies and classified as feral (n=10), semi-feral (n=10) and domesticated (n=9) based on management conditions and analyzed using 16S rRNA sequencing. Feral horses had higher levels of Methanobacteria while domesticated horses had a high abundance of members from the phyla Proteobacteria and Tenericutes. The semi-feral group had intermediate levels of the taxa within the feral and domesticated groups and had the highest within group variation in terms of bacterial diversity. Lastly, when looking at microbial functionality, this revealed that energy metabolism was increased in the feral group while lipid and amino acid metabolism was increased in the domesticated group, revealing how diet differences can affect the microbial composition (Bull et al., 2024).

Although there are feral equine populations living in the United States, there are limited to no published studies assessing the microbiota within these populations. Feral populations of horses can be found in the western US such as in Nevada and in states within the eastern US such as Virginia, North Carolina and Maryland. By assessing the microbiota between feral and domesticated population of horses, this can provide insight into how diet and management practices affect the equine microbiota.

### 1.7. Effects of Pre- and Probiotic Use on the Equine Microbiome

Pre- and probiotic use for horses is common due to their perceived benefits on equine health, such as supporting digestion, reducing stress associated with intestinal issues and enhancing performance in athletic horses (Cooke et al., 2023). Previous studies have shown that prebiotics and probiotics influence digestibility both *in vivo* and *in vitro* and can help stabilize the microbiome in the equine large intestine, however studies done within this area have shown

conflicting results (Garber et al., 2020; Grimm et al., 2016). Therefore, there seems to be more to learn about the effectiveness of these supplements on the microbiome and overall gastrointestinal health within horses.

Prebiotics are substrates utilized by host microorganisms to confer a health benefit (Garber et al., 2020; Gibson et al., 2017). Common prebiotics used in animal feeds include inulin, fructo-oligosaccharides, manno-oligosaccharides, yeast fermentation products and others. Oftentimes, these supplements are marketed to support digestion, metabolism, growth and immunity (Cooke et al., 2023). However, there are limited studies on the efficacy of prebiotics on hindgut health, with conflicting results.

In a recent study utilizing a prebiotic nutritional supplement, Adams et al. (2022) collected fecal samples from Thoroughbred horses in full training to assess differences of the fecal microbiome. In a pilot study using 4 racehorses, the microbiome was dominated by the phylum Bacteroidetes followed by Firmicutes. After supplementation for 2 months, abundances of Bacteroidetes decreased while Firmicutes increased, which resulted in an increased F/B ratio. During the clinical trial within the study, the treated/supplemented horses had a lower abundance of Bacteroidetes when compared to the control group. Additionally, supplemented horses were more likely to have an increase in Firmicutes and Actinobacteria compared to control horses. Although this study did have limitations, it does appear that when utilizing a prebiotic supplement, the fecal microbiome resembles those microbiomes seen in horses primarily forage fed or pasture grazed (Adams et al., 2022).

Probiotics are live microorganisms that confer a health benefit on the host when administered in appropriate amounts according to the Food and Agricultural Organization of the United Nations (Food et al., 2006). Microorganisms commonly used as probiotics include yeast, such as

*Saccharomyces cerevisiae* and bacteria such as *Lactobacillus* and *Bifidobacterium*. In the United States, probiotics can be classified two ways: as a drug, in which Food and Drug Authority (FDA) approval is needed or as a feed supplement that is "generally regarded as safe" (GRAS), in which supplement producers do not need to go through FDA approval and GRAS classification is based on information provided by the producer (Kauter et al., 2019; Schoster, 2018). Similar to prebiotics, there are limited studies on the efficacy of probiotics on the equine microbiome and gastrointestinal health.

In a study assessing the microbiota of neonatal foals to reduce diarrhea incidence, a multi-strain probiotic consisting of strains of *Lactobacillus* and *Bifidobacterium* was utilized. There were no differences found between the control and treatment group of relative abundance of the 2 genera that were within the probiotic supplement, although there was an enrichment of *Lactobacillus* in the treatment group at 6 weeks. Based on the study, in which there was a lack of clinical effects of probiotic supplementation seen in the study population, this specific supplement utilizing these bacterial strains may not prevent diarrhea from occurring in foals (Schoster et al., 2016). These results conflict with another study (Tanabe et al., 2014), in which administration of a probiotic consisting of *Lactobacillus* and *Bifidobacterium* decreased diarrhea incidence in neonatal Thoroughbreds. However, these conflicting results could be due to utilizing different strains in the probiotic supplement.

Given the limited regulation of pre- and probiotics, especially within the United States (Zoumpopoulou et al., 2018), these supplements need to be continued to be evaluated within horses to see if they truly affect gastrointestinal health.

### 1.8. Main Concepts used in Microbial Ecology

In order to understand the intricacies of assessing the microbiome, a number of different terms are used to describe concepts used in microbial ecology. These important concepts are outlined below (Table 1.1) (Costa & Weese, 2018).

When studies are investigating the taxonomic classification of bacterial communities and which bacteria are present, the term "microbiota" should be used and if a study is investigating the overall genetic makeup or function potential, the term "microbiome" should be utilized. Another way to look at and define "microbiota" and "microbiome" is that it is the different microorganisms associated within a particular space and it is the corresponding entity of genetic material, respectively (Kauter et al., 2019; Ursell et al., 2012).

When changes in beta diversity are significant and changes in membership and structure, this allows for some inferences. If there are changes in membership but not structure, this implies that changes are likely from rare members being added or dropped. However, if the structure changes but membership remains the same, this implies that there are changes in the numbers of existing members (Costa & Weese, 2018).

If a study wants to assess the entire horse population within a region, state or province, a larger sample size needs to be taken in order to better estimate the population. If all horses within the population can be accounted for, this is the absolute richness. This absolute richness is similar to microbiota assessment, in which these indices allow for estimates that are influenced by sample size and how representative the sample population is compared with the overall studied population. Counting each horse allows for description of the absolute abundance (number) and relative abundance (percentage). Since absolute abundance is difficult and not feasible to determine, description of individual members is based on relative abundance (Costa & Weese, 2018).

*Table 1.1. Terms and definitions used in microbial ecology. (Adapted from (Costa & Weese, 2018))*

| Term | Definition |
|---|---|
| Microbiota | All microorganisms of a particular environment |
| Microbiome | All microorganisms with their genetic material and their interaction with an environment |
| Alpha-diversity | Describes the characteristics of individual samples (e.g., richness, evenness and diversity) |
| Richness | Total number of taxa (e.g., species or genera, families, phyla) present in an environment, either through direct measure (observed richness) or through calculations to estimate true richness that would have been detected if the entire population had been studied (estimated richness); Alpha diversity indices can be described and compared between groups |
| Evenness | Distribution of species (e.g., prevalence or relative abundance of each population within a community) |
| Diversity | Mathematical equation that takes into account richness and evenness (i.e., it quantifies how equal a microbial community is) |
| Beta-diversity | Comparisons between samples or groups assessed in a variety of ways with different indices; Compares the overall composition of the microbiota, typically based on membership or community structure |
| Membership | Members (e.g., species) that are or aren't present |
| Structure | Broader comparison that takes into account the members that are or aren't present, and their relative abundance |

### 1.9. *In Vitro* Fermentation

*In vitro* fermentation models are beneficial to utilize to assess the amount and types of gases and other metabolites, such as volatile fatty acids, produced. Thus, utilizing *in vitro* models can help quantify the type of fermentation occurring within the hindgut in horses. *In vitro* methods

are commonly used in ruminant species to assess digestibility and are highly correlated to values determined *in vivo* (Goldman et al., 1987). Rumen or cecal fluids are commonly used as an inoculum for *in vitro* methods, however this technique is limited in equine research as cannulated animals are not as readily available compared to ruminants. Despite this, feces have been successfully used as a source of microbial inoculum to measure digestibility using an *in vitro* method (Earing et al., 2010; Lowman et al., 1999). Thus, several studies have utilized fecal materials as the inoculum to study digestibility in horses.

Early studies have found that volatile fatty acids can help meet energy needs, in which Glinsky et al. (1976) found VFA production can meet up to 30% of maintenance energy needs (Glinsky et al., 1976) while Vermorel et al. (1997) determined that 80% of energy needs can be met when horses are consuming a hay diet (Vermorel et al., 1997). Additionally, when looking at acetate specifically, Hintz et al. (1971) found that acetate was produced at ~74% in cecal or colonic fluid when horses were on an all-roughage diet (Geor et al., 2013; Hintz et al., 1971). In a more recent study, Biddle et al. (2013) utilized an *in vitro* model to assess patterns of metabolic end products and the microbial community composition when enriched with lactate and/or starch to emulate an *in vivo* laminitis induction. Lactate enriched treatment resulted in an initial increase followed by declining lactate levels while acetate, propionate and butyrate increased over time. In the starch enriched treatment, acetate, propionate and butyrate stayed relatively the same throughout the 48 hours (Biddle et al., 2013).

Gandarillas et al. (2021) assessed gas production and volatile fatty acid concentrations of working equine diets, in which fermentation was assessed over a 96-hour period using different dietary treatments. pH at the end of the period was significantly greater in forages versus concentrates. Although concentrations of acetate, propionate and butyrate did not differ between

concentrates and forages as well as within forages or concentrates, forage fermentation did result in a higher acetate to propionate ratio. Gas production and VFA concentrations were greater in concentrates versus forages as well as faster fermentation rate was observed, which is in agreement with other studies (Elghandour et al., 2014; Gandarillas et al., 2021).

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## PART II: IRON & INFLAMMATORY CYTOKINES

### 1.10. Introduction

Iron is a chemical element, denoted by the symbol Fe, and its atomic number is 26 (Boldt, 1999). Iron is a transition metal in which it can donate and accept electrons, thus participating in oxidation-reduction reactions that play important roles in biological processes (Dev & Babitt, 2017). It is one of the key components of the Earth's core, comprising about 5% (Pantopoulos et al., 2012), with soil also being enriched in this element (Boldt, 1999). Iron commonly exists in two different states of oxidation: a ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) form. The ferric form of iron is poorly water-soluble and stable while the ferrous form is water soluble but highly reactive.

Iron is also considered an essential element for all living organisms (Abbaspour et al., 2014; Lieu et al., 2001). When humans and/or animals consume iron, it exists in 2 dietary forms: heme and non-heme. Common sources of heme iron are from consumption of meat, poultry and fish products while sources of non-heme iron include cereals, legumes, fruits, vegetables and others. Heme iron is more bioavailable at 15-35% as it is derived from hemoglobin and myoglobin of animal food sources versus non-heme at 2-20%. Additionally, absorption of non-heme iron can be influenced by other food components (e.g., phytate, ascorbic acid) while this has a lesser effect for heme iron (Abbaspour et al., 2014; Ems et al., 2017; Hurrell & Egli, 2010). In herbivores like our equines, they will be consuming non-heme iron as a primary dietary iron source within their diet.

Within the body, iron serves important physiological functions such as oxygen transport, cellular respiration, electron transport and is a component of heme (e.g., hemoglobin, myoglobin) (Dev & Babitt, 2017; Ems et al., 2017; Schryver, 1990), thus the reason it is an

essential element for living organisms. Absorption of iron is tightly regulated within the body in order to achieve iron homeostasis and daily losses are minimal, with about 1-2 mg of iron lost in humans due to blood loss and epithelial desquamation (Dev & Babitt, 2017; Yiannikourides & Latunde-Dada, 2019). Iron homeostasis is achieved through key hormones (e.g., hepcidin), proteins (e.g., ferritin) and organs (e.g., liver), which will be discussed in further detail.

This review will cover iron metabolism and homeostasis and the key players involved. Additionally, it will discuss iron requirements in horses and common dietary sources as well as previous literature related to iron metabolism in horses as well as other species when applicable. Finally, inflammatory cytokines and the potential correlation of inflammation, insulin dysregulation and iron will be discussed.

## 1.11. Iron Metabolism

### *1.11.1. Iron Distribution*

Iron is distributed in several areas throughout the body and is generally similar across humans, horses and other mammals. The majority of iron is found within hemoglobin in red blood cells, corresponding to about 65% or ~2 g of iron in humans (Dev & Babitt, 2017), thus greatly enhancing blood's oxygen carrying capacity (Kamerling & Tobin). Typically, the bone marrow will be the largest iron consumer as a billion iron atoms daily are necessary for red blood cell production (Andrews & Schmidt, 2007). The second most prevalent distribution of body iron is as storage iron compounds (e.g., ferritin, hemosiderin), comprising 25-26%, however most stored iron will likely be in the form of ferritin which can then be utilized when needed within the body (Andrews & Schmidt, 2007). When iron is stored, it is stored as ferritin in order to minimize circulating iron (Roemhild et al., 2021). Ferritin is a well-known iron storage protein, primarily synthesized and stored in the liver by hepatocytes. However, other cells can synthesize

ferritin, just to a lesser degree (Anderson & Shah, 2013; Theil, 1987). Ferritin has a shell-like structure formed by 24 chains with a heavy and light chain, which can store up to 4500 iron atoms (Camaschella et al., 2020). Storing iron as ferritin allows for tighter control of iron delivery to the basolateral exporters, known as ferroportin, ultimately mediating systemic iron delivery, especially into the bloodstream (Dev & Babitt, 2017; Donovan et al., 2000). Ferritin does have only a life span of a few days and is in a constant state of turnover due to this (Kamerling & Tobin). Measuring serum ferritin levels is thought to be the best and most convenient way to measure iron body stores both in humans (Abbaspour et al., 2014) and horses. Smith et al. (1984) found that mature horses have serum ferritin levels with a mean ± standard deviation of 152 ± 54.6 ng/ml and a range of 70 - 250 ng/ml, which is correlated to total body iron stores (Smith et al., 1984). Hemosiderin is the other storage form of iron, an insoluble, iron-rich material primarily found in lysosomes (Theil, 1987). Despite hemosiderin being iron-rich, iron is less readily available to be released for needs within the body (Abbaspour et al., 2014).

Iron is also distributed in the body as myoglobin, comprising about 3-5%, ~300 mg in humans, which can be found within the muscle, heart or other organs (Dev & Babitt, 2017). In horses, it has been found that iron in myoglobin is found within the muscle at 0.01 g iron per kg of muscle (Kamerling & Tobin). Finally, less than 0.1% of total body iron is distributed as transferrin, corresponding to about ~3 mg in humans, however this is dynamic as daily turnover occurs frequently due to the high demand for erythropoiesis (i.e., red blood cell production). Transferrin is a transporter and the primary form of iron found within the bloodstream. Transferrin can hold up to two iron atoms, in which it captures iron released from intestinal enterocytes or macrophages and can then deliver iron to various tissues (Abbaspour et al., 2014; Dev & Babitt, 2017).

*1.11.2. Iron Absorption*

A human's daily diet contains 10-20 mg of iron but minimal iron is actually absorbed, about ~1-2 mg. However, iron absorption can range from 5-35% depending upon the form of iron as well as specific circumstances (e.g., iron deficiency) (McDowell, 1992). Additionally, iron absorption is controlled by the existing iron levels within the body. Iron absorption primarily occurs within the small intestine, specifically in the duodenum and proximal jejunum through enterocytes. Once iron is absorbed, it can either be stored as ferritin within the enterocytes or enter the circulation to be transported to various tissues via the plasma-protein transport transferrin (Yiannikourides & Latunde-Dada, 2019), as detailed above.

Non-heme iron is mainly in the oxidized, ferric ($Fe^{3+}$) form and must be reduced to the ferrous ($Fe^{2+}$) form prior to intestinal uptake. In the proximal duodenum, gastric acids lower the pH and the reduction of ferric to ferrous ions occurs, facilitated by ferrireductases in the apical membrane (e.g., duodenal cytochrome B) and dietary ascorbic acid (Dev & Babitt, 2017; McKie et al., 2001). However, the production of gastric acid is impaired due to drugs like Omeprazole being present, which act as an acid pump inhibitor, thus reducing iron absorption (Abbaspour et al., 2014). Additionally, if there is excess ferrous iron, this can lead to the formation of reactive oxygen species (ROS) through the Fenton reaction, which can result in cellular damage and death (Dixon & Stockwell, 2014; Roemhild et al., 2021).

Once iron has been reduced to the ferrous ($Fe^{2+}$) form, it can then be transported into the enterocytes by divalent metal transporter 1 (DMT1) (Figure 1.2). This transporter also transports other minerals (e.g., zinc and copper) across the brush border and it has been shown that these minerals will compete for absorption. For example, iron supplementation can reduce serum zinc

in pregnant women (Hambidge et al., 1983) and both serum and liver zinc in horses (Lawrence et al., 1987), thus these two minerals are considered antagonistic.

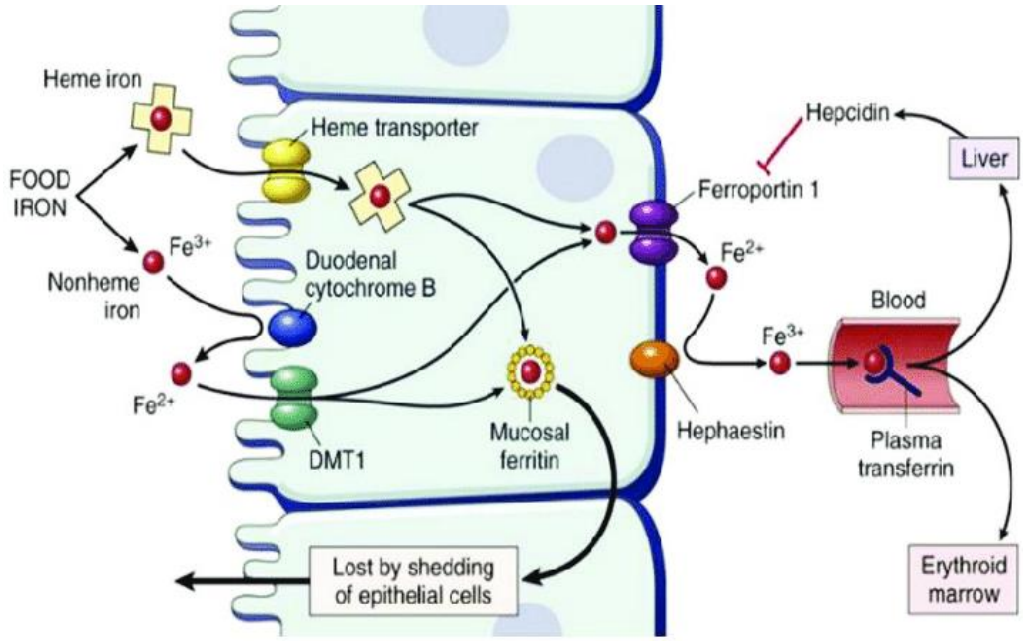


*Figure 1.2. Depiction of iron absorption across the cellular membrane within the small intestine. (Source: (Dasa & Abera, 2018))*

After iron is absorbed through DMT1, the fate of it will depend upon the iron status within the body. Overall, iron can be used for metabolic processes within the cells, it can be stored as a storage protein (e.g., ferritin) or it can be exported across the basolateral membrane to be delivered to specific tissues (Dev & Babitt, 2017). If demand for iron is low, iron will primarily be stored as ferritin, the main intracellular iron storage protein. However, if demand for iron is high, the absorbed iron will be transported across the basolateral membrane and into the bloodstream. This is accomplished by ferroportin (FPN1), which regulates how much iron goes into circulation and is ultimately made available within the body (McKie et al., 2000; Yiannikourides & Latunde-Dada, 2019). The expression of FPN1 is regulated by the hormone

hepcidin, which is perceived as the master regulator of iron homeostasis. As discussed previously, if there is free circulating iron in the bloodstream, this will be picked up and transported where it is needed via the transporter protein transferrin.

*1.11.3. Regulation of Iron Homeostasis*

Due to the importance of iron in various physiological functions, its uptake, utilization, transportation and storage all need to be carefully balanced to maintain homeostasis (Abbaspour et al., 2014; Lieu et al., 2001). Homeostasis is regulated at both the cellular and systemic level through a number of different key players (e.g., hepcidin, ferritin, etc.) (Figure 1.3).

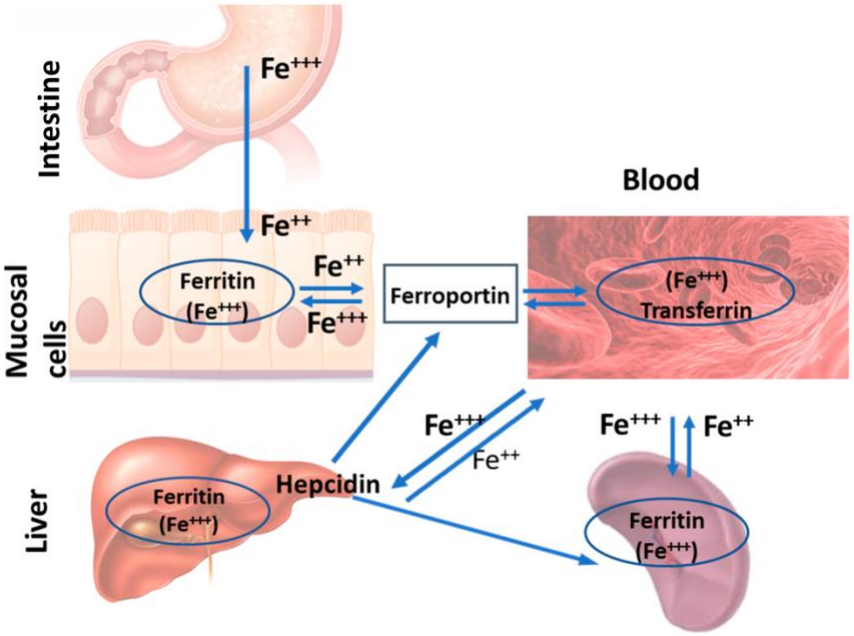


*Figure 1.3. Main tissues, hormones and proteins involved in systemic iron homeostasis. (Source: (Yiannikourides & Latunde-Dada, 2019))*

At the cellular level, iron must be regulated to ensure there are adequate amounts of iron for essential functions while preventing the formation of ROS due to free iron. Cellular homeostasis is achieved two ways: ferritin and iron regulatory proteins. Ferritin is the 1st mechanism, acting as a storage mechanism for excess iron and allowing cells to mobilize it when

needed. Iron regulatory proteins (IRP) are the 2nd mechanism, which will alter their activity according to cellular iron levels. There are two types of IRPS in mammalian cells: IRP1 and IRP2 (Andrews & Schmidt, 2007; Zhang et al., 2014).

Systemically, iron homeostasis is regulated via hepcidin, which is thought to be the master regulator (Abbaspour et al., 2014; Camaschella et al., 2020; Dev & Babitt, 2017; Yiannikourides & Latunde-Dada, 2019). Hepcidin in its biologically active form is a 25-amino-acid peptide hormone, primarily produced by hepatocytes in the liver (Camaschella et al., 2020; Grimes et al., 2012). However, it can also be produced by pancreas (specifically, in β-cells), blood stem cells and the heart (Andrews & Schmidt, 2007; Ilyin et al., 2003). Across species, the amino acid sequence of hepcidin has remained highly conserved (Fry et al., 2004). Hepcidin was only recently discovered by researchers in 2000-2001 as a novel peptide, first termed liver-expressed antimicrobial peptide-1 (LEAP-1) (Grimes et al., 2012; Krause et al., 2000). The discovery of this peptide hormone was a breakthrough in researchers' understanding of how iron is systemically regulated and potential issues with iron disorders (Camaschella et al., 2020).

The main role of hepcidin is to control the amount of iron within the circulation through a negative feedback mechanism and by acting in different target tissues. Regulation of hepcidin transcription in the hepatocytes and subsequent expression will depend upon circulating and tissue iron (Camaschella et al., 2020). In instances of high iron (e.g., increased serum iron, iron overload and inflammation (Andrews & Schmidt, 2007; Nicolas et al., 2002)) hepcidin expression will be increased. Hepcidin will then act by binding to ferroportin, an iron transporter found on enterocytes within the duodenum or on macrophages, and cause it to be degraded and internalized, thereby blocking iron from being released into the bloodstream (Figure 1.4). However, in instances of low iron (e.g., iron deficiency, increased erythropoietic demand),

hepcidin expression will be decreased, ultimately allowing iron into the circulation via ferroportin and promoting iron availability (Dev & Babitt, 2017). Through hepcidin, systemic homeostasis of iron is tightly regulated in order to prevent iron overload, deficiency and the potential production of reactive oxygen species.

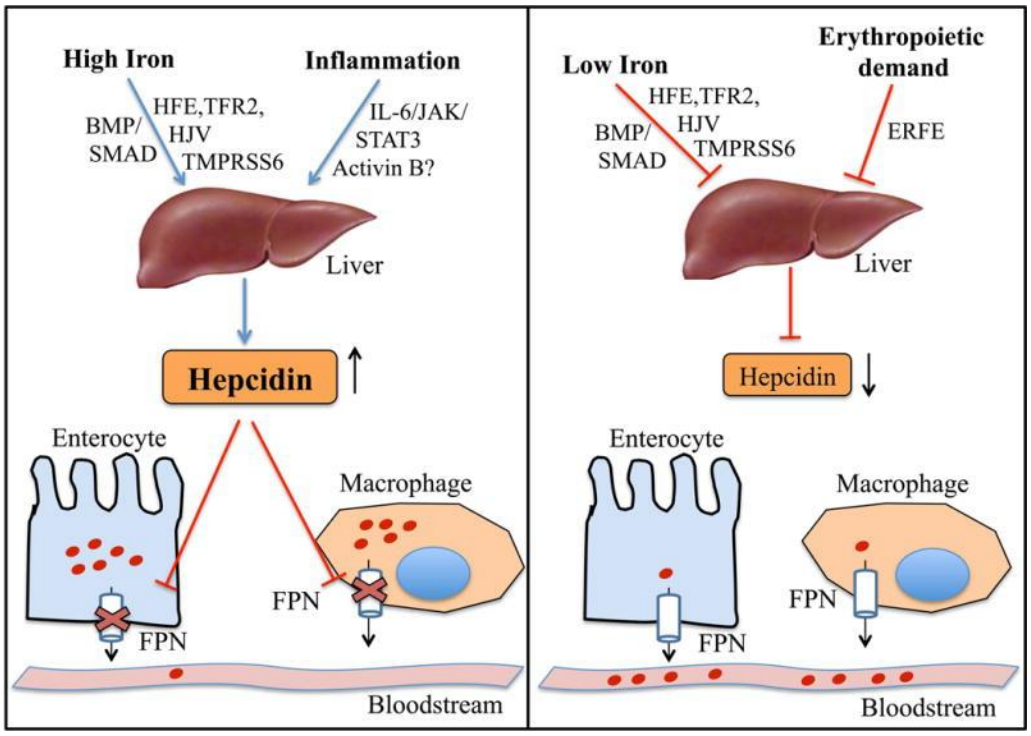


*Figure 1.4. Depiction of systemic iron homeostasis by hepcidin, a peptide hormone produced in the liver. (Source: (Dev & Babitt, 2017))*

*1.11.4. Iron Excretion*

Iron is unique from other minerals as there is no active excretion mechanism from the body. Therefore, total body iron is regulated at the point of absorption. As mentioned previously, the bone marrow is the largest consumer of iron (~20-25 mg in humans) in order to meet the needs for red blood cell production (Yiannikourides & Latunde-Dada, 2019). With ~1-2 mg of iron actually being absorbed from the duodenum this accounts for the equal loss of iron from epithelial desquamation, which can be found in the feces, and blood loss in order to maintain iron

balance. Iron can also be lost through sweat or via the urine, although this is minimal due to iron being bound to transferrin (Dev & Babitt, 2017; Yang et al., 2003). Additionally, menstruation can also cause moderate losses of iron in order to support reproduction in primates and egg-laying birds (Finch et al., 1978; Grimes et al., 2012) and in women, excessive blood loss during menstruation has been found to be a cause of iron deficiency (Abbaspour et al., 2014). Finally, with the lack of an active iron excretion mechanism, the body cannot increase excretion of iron in instances of iron overload (Grimes et al., 2012; Hentze et al., 2010), which could cause potentially harmful issues.

## 1.12. Iron in Horses

### *1.12.1. Dietary Sources and Requirements*

Common dietary sources of iron for horses include forages, grains, by-product ingredients and vitamin-mineral supplements. Overall, a common equine diet will contain >100 mg per kg DM of iron (Geor et al., 2013). Forages and by-products will contain 100-250 mg per kg DM with grain containing < 100 mg per kg DM, per the National Research Council (NRC) (NRC, 2007). It has been reported that hay will often contain higher iron levels than what the NRC reports, with Richards and Nielsen finding in the Equi-Analytical Feed Composition Database (https://apps.dairyone.com/feedcomposition/eq/) a mean Fe of 212 mg/kg and range of 31-3760 mg/kg and 446 mg/kg and 47-6360 mg/kg for grass and legume hay, respectively (Richards & Nielsen, 2018). These higher iron levels seen are likely due to contamination from both soil and steel machinery (Geor et al., 2013). Vitamin-mineral supplements can contain variable amounts of iron, with one popular equine example being Red Cell (https://www.horsehealthproducts.com/all-products/red-cell-vitamin-iron-mineral-supplement), which provides 300 mg of iron per fluid ounce in the form of ferric sulfate. Another example of

an equine iron-specific supplement is Finish Line Iron Power (https://finishlinehorse.com/product/iron-power/), which states it can be used to prevent iron deficiency anemia and provides 560 mg of iron per serving in the form of ferrous sulfate. However, claims from supplement companies that their product will prevent iron deficiency anemia should be met with caution, as generally iron deficiency is not a concern in foals or mature horses as long as they have access to soil (NRC, 2007).

Information on endogenous losses of iron hasn't been reported in horses, likely due to how tightly regulated iron is as well as the lack of active excretion mechanisms. Therefore, requirements have remained unchanged since the 1989 publication of the NRC Nutrient Requirement of Horses (NRC, 1989, 2007). Currently, daily iron requirements are estimated to be 50 mg/kg DM for growing foals, pregnant and lactating mares and 40 mg/kg DM for mature horses (NRC, 2007), equating to 400 mg daily for a 500 kg horse at maintenance. In performance horses, it has been proposed to provide higher daily iron due to the increased turnover of red blood cells (Geor et al., 2013). Additionally, iron loss through sweat has been assessed in exercising horses, however this was reported to be 0.6% of intake, thus this would not call for a cause in increased daily iron for performance horses so requirements for performance horses are currently set at the maintenance level (40 mg/kg DM) (Inoue et al., 2005; NRC, 2007). Therefore, exercising horses should meet their iron requirements through increased DM feed intake, although it has been reported that racing Thoroughbred trainers will supplement their horses with an iron-specific supplement to prevent anemia and to boost red blood cells in order to enhance performance (McLean et al., 2022).

Maximum tolerable concentrations for iron have not been established in horses (NRC, 2006, 2007). Iron toxicity is also rare in horses, although foals are more susceptible to toxicity

and ferrous fumarate has been found to be toxic in mature horses (Arnbjerg, 1981; NRC, 2007). Currently, the maximum tolerable concentration is set at 500 mg Fe per kg of ration based on data from other species (NRC, 2006). However, it is likely that this established maximum tolerable concentration is not accurate in horses (NRC, 2007). Richards and Nielsen reported that of the 5837 hay samples they analyzed from the Equi-Analytical Feed Composition Database (Ithaca, NY), 12% contained iron at or over the maximum tolerable threshold while 0.2% contained less than 40 mg Fe/kg, meaning most hay is supplying iron in excess, likely due to soil and steel machinery contamination (Geor et al., 2013; Richards & Nielsen, 2018). Therefore, it seems plausible that iron supplementation is not necessary as daily iron requirements should be being met through their diet. Additionally, there is little evidence that supplementation actually improves hemoglobin and/or oxygen-carrying capacity (Kirkham et al., 1971) despite these perceived benefits by Thoroughbred trainers (McLean et al., 2022). However, the bioavailability of an iron supplement could be playing a role as some sources of iron contain more elemental iron than others, for example, ferrous sulfate only contains 20% elemental iron.

*1.12.2. Previous Iron Metabolism Literature in Horses*

Although there is limited research in horses related to iron metabolism, previous studies have yielded interesting results, providing further insight on the intricacies of iron metabolism in horses. Pearson and Andreasen evaluated the effects of excess iron via oral iron supplementation in ponies. Ponies (n=4) were fed 50 mg of iron/kg BW in the form of ferrous sulfate daily for 8 weeks, estimating study subjects were receiving 2500 μg/g of feed of elemental iron since ferrous sulfate contains 20% elemental Fe. Blood and tissue samples were collected to evaluate various blood iron indices and hepatic iron concentrations. Serum iron and ferritin concentration increased throughout the study as well as hepatic iron concentrations, however no histologic

lesions or adverse clinical effects were found (Pearson & Andreasen, 2001). However, another study found that serum iron (as well as hemoglobin and hematocrit) did not increase despite supplementing ponies with high levels of iron (Lawrence et al., 1987). Based on the results of the Pearson and Andreasen study, iron toxicosis is unlikely to occur over 8 weeks, but it is important to note that an inorganic source of iron was used in the form of ferrous sulfate (Pearson & Andreasen, 2001).

Interestingly, the effects of iron supplementation on the equine fecal microbiome were recently assessed (Arantes et al., 2023). Microbiome and metabolism are closely related and the microbiome does play an important role in gastric health. Additionally, low iron has been shown to reduce bacterial DNA and RNA synthesis, resulting in bacterial morphology changes (Khasheii et al., 2021). Treated horses were supplemented with 720 ppm of iron via ferrous sulfate monohydrate for 15 days, with fecal samples taken throughout the study as well as 30 days from the beginning of iron supplementation. Fe supplementation caused no change in the fecal microbiota composition overall compared to control horses, although there was increased alpha diversity after supplementation. Thus, it seems that iron supplementation results in only minor changes in low-abundant bacteria and will increase diversity within individuals, however the relationship between iron and the microbiota could be investigated further with different doses as well as dietary iron sources (Arantes et al., 2023).

Due to iron's importance as a component of hemoglobin and myoglobin and its involvement in oxygen transport, studies in exercising horses have been done to assess how iron metabolism is affected by exercise. Previous studies in humans have shown iron loss through sweat (Paulev et al., 1983), decreased iron absorption, serum iron concentrations and body iron stores (Nachtigall et al., 1996; Nickerson et al., 1985), however research on iron metabolism in

exercising horses is limited. Exercise on a high-speed treadmill decreased both serum iron and total iron binding capacity (TIBC), with TIBC being significantly different before exercise and the 3rd week of exercise. Loss of iron in sweat did not differ between different weeks (and intensity) of exercise. However, iron balance and apparent absorption significantly increased due to exercise, likely to compensate for the breakdown of red blood cells occurring due to exercise (Inoue et al., 2005). In a study assessing show jumping horses (n=10) over 3 days of competition and 1 day after competition ended, results showed significant effects of exercise on hemoglobin and hematocrit, serum ferritin, serum iron and TIBC, with these concentrations generally increasing throughout the subsequent days of competition (Piccione et al., 2017). Thus, this indicates the potential effects of hemolysis on exercise and iron indices. Plasma ferritin has also been evaluated in exercising horses, with ferritin increasing even with moderate exercise. Additionally, the increase in plasma ferritin was greater when exercise duration and intensity increased, indicating that ferritin is useful to assess iron stores that can be mobilized (Hyyppä et al., 2002).

Although hepcidin has been discovered to be the master regulator of iron homeostasis, little research on this peptide hormone has actually been done in horses, especially assessing serum hepcidin (Grimes et al., 2012). Equine hepcidin mRNA has been identified and sequenced and found to be significantly higher in hepatic tissue versus others (Oliveira Filho et al., 2010). Additionally, hepcidin mRNA expression has been shown to be upregulated when inflammation was induced using Freund's complete adjuvant (Oliveira-Filho et al., 2014) and lipopolysaccharide (Oliveira-Filho et al., 2012). In a study of native Mongolian horses (n=26), the anti-hepcidin effect of pentosan polysulfate (PPS), which is used to treat mild or early-stage osteoarthritis, was assessed. Before the PPS treatment began, serum hepcidin concentrations

across the treated and placebo horses was 110.1 ± 40.2 ng/ml. However, in horses treated with PPS, hepcidin concentrations were lower than placebos over the study period, indicating that PPS has an anti-hepcidin effect and could improve iron availability, which could improve oxygen transport (Wijekoon et al., 2022).

Hepcidin and other iron indicators have also been checked during reproductive cycles in horses as greater mobilization of iron stores is needed to support fetal development. In cycling mares, blood samples were collected at various days (-5, 9, +5, +16) of the estrous cycle. Serum iron was found to be lower on days +5 and +16 versus -5 and 0 while ferritin and hepcidin were found to be higher on day +16 versus -5 and +5. Interestingly, there was a decrease of serum hepcidin at day 0 of the cycle. In the follicular phase, during which the follicle is developing, this is characterized by a decrease in hepcidin and increases in serum iron, ferritin and estradiol. Meanwhile, in the luteal phase, during which the corpus luteum is formed, serum iron and ferritin decreased while hepcidin and progesterone increased (Satué, Fazio, La Fauci, et al., 2023). Overall, the study revealed relationships between reproductive hormones and iron metabolism indicators. In another study assessing markers of iron homeostasis but in pregnant mares, it was found that serum iron and ferritin increased while hepcidin decreased throughout the 11 months of pregnancy. Additionally, progesterone was positively correlated with hepcidin, suggesting an important relationship between these two hormones (Satué, Fazio, Cravana, et al., 2023).

### 1.13. Inflammatory Cytokines: TNF-α

Cytokines are a broad term used to describe small and nonstructural proteins that serve as intercellular signaling peptides (Cannon, 2000; Dinarello, 2000). Cytokines can be classified as either pro- or anti-inflammatory cytokines, with a broader classification being inflammatory

cytokines. The primary function of anti-inflammatory cytokines is to inhibit inflammation. Inflammatory cytokines within this classification include IL-1 antagonist, IL-4, IL-6, IL-10 and others (Opal & DePalo, 2000). The primary function of pro-inflammatory cytokines is to promote inflammation and these cytokines include several types of interleukins (IL) and the family tumor necrosis factor (TNF). Some key pro-inflammatory cytokines include IL-1, IL-6 and TNF-α (Turner et al., 2014). Hepcidin, a peptide hormone that is the master regulator of iron homeostasis, is also thought to be regulated by inflammatory cytokines, specifically IL-6 (Camaschella et al., 2020), with IL-6 upregulating hepcidin transcription (Grimes et al., 2012; Hentze et al., 2010), thus showing the interconnectedness of iron homeostasis and inflammatory cytokines within the body (Abbaspour et al., 2014). This section of the review will mainly focus on the pro-inflammatory cytokine TNF-α. TNF-α has several physiological functions, including causing tumor cell necrosis and apoptosis and is a key mediator of acute and chronic inflammatory responses. It is primarily produced by macrophages, although other cells may be able to produce it at lower levels (Chu, 2013).

Inflammation and obesity are interrelated as obesity is considered a chronic mild inflammatory state (Dandona et al., 2004). With the correlations between obesity, inflammation, insulin resistance and subsequent metabolic issues, TNF-α levels have been assessed in order to better understand its role. The role of TNF-α in mediating insulin resistance has been shown in studies utilizing rodent models, in which TNF-α levels were elevated in obese and insulin-resistant rodents (Hotamisligil et al., 1993; Sethi & Hotamisligil, 2021; Vick et al., 2007). Therefore, it appears that TNF-α does play a potential role in obesity and other metabolic issues and this has been explored in horses in the previous literature.

In a study using 60 mares of various ages and measuring obesity by the variables % fat (determined by ultrasound) and body condition scores (BCS) (Henneke et al., 1983), inflammatory cytokines and insulin sensitivity was assessed. In older mares (20 years or older), TNF-α and insulin sensitivity were inversely related and increased BCS and % fat resulted in increased TNF-α expression. The results of this study indicate associations between elevated inflammatory cytokines and obesity in horses (Vick et al., 2007). As increasing age has been shown to be associated with chronic inflammation (dubbed "inflamm-aging") (Adams et al., 2009; Franceschi et al., 2000), a study in older horses was done to evaluate the effects of adiposity on inflammatory cytokine levels. When body weight and fat were reduced in older horses through dietary restriction, both serum TNF-α protein levels and percent of TNF-α positive lymphocytes and monocytes significantly reduced. Therefore, age-related obesity or "inflamm-aging" is likely playing a role in inflammatory cytokine production within horses (Adams et al., 2009).

Another study looking at obese ponies and ponies with equine metabolic syndrome (EMS) measured histological and serum IL-6 and TNF-α levels. Adipose tissue samples from EMS ponies were infiltrated with macrophages and lymphocytes compared to obese ponies. Additionally, serum levels of both IL-6 and TNF-α were elevated in ponies with EMS, with this group having a mean serum TNF-α concentration of 1.97 ug/mL versus 1.21 ug/mL for obese ponies. Based on these results, measuring serum pro-inflammatory cytokines may be a useful diagnostic tool for diagnosing EMS (Basinska et al., 2015). Finally, exercise may also influence inflammatory cytokines, with human studies finding increases of cytokines such as TNF-α and IL-6 during and after exercise, however these increases may be affected by duration, type and intensity of exercise (Ostrowski et al., 1998; Ostrowski et al., 1999). In a study by Liburt et al.

(2010), inflammatory markers were assessed both in the muscle and blood of horses undergoing an incremental exercise test on a high-speed treadmill. The results of this study indicated that TNF-α increased rapidly in the muscle following exercise but had a delayed increase in blood post-exercise. Thus, it does appear that exercise induces inflammatory cytokine expression in the blood and muscle within horses (Liburt et al., 2010).

### 1.14. Correlation between Iron, Obesity and Insulin Dysregulation

Iron is necessary within the diet in order to support essential physiological functions but iron has also been implicated in affecting glucose and lipid metabolism, in which insulin causes uptake of iron by adipocytes (Dev & Babitt, 2017; Le Guenno et al., 2007; Tanner & Lienhard, 1987). This has primarily been shown in humans and/or rodent models, wherein high body iron stores, determined by serum ferritin, has resulted in an increased risk of insulin resistance (Dev & Babitt, 2017; Ford & Cogswell, 1999). In a study with Wistar rats fed a high fat/high energy (HF-HE) diet, the insulin-resistance index was higher in the HF-HE group and hepatic hepcidin mRNA expression was lower in the HF-HE group. The study results indicate that insulin resistance could lead to a downregulation of hepcidin expression and that assessing this peptide hormone could be useful when further investigating iron overload and metabolic syndromes (Le Guenno et al., 2007).

Obesity is considered a chronic, mild inflammatory state and therefore can have subsequent effects on iron homeostasis. Previous research has shown that obesity is associated with increased hepcidin, serum ferritin and increased iron stores in the body but simultaneous decreases in serum iron (Jehn et al., 2004; Moore Heslin et al., 2021; Vuppalanchi et al., 2014). Additionally, hepcidin is produced in the adipose tissue and has been found to be expressed higher in this tissue in obese humans. Subsequently, IL-6, a prominent pro-inflammatory

cytokine, promoted hepcidin expression in this study (Bekri et al., 2006). As hepcidin is considered an acute phase protein, it seems that inflammatory cytokines like IL-6 and IL-1 will increase hepcidin expression, which ultimately results in increased serum hepcidin concentrations in instances of acute and chronic inflammatory states like obesity (Grimes et al., 2012; Lee et al., 2005; Nemeth et al., 2003; Theurl et al., 2009).

Despite the research done in humans of the potential correlation between iron, obesity and subsequent metabolic issues (Moore Heslin et al., 2021), surprisingly little work has been done in horses. Obesity is rampant in our equine population and can lead to numerous health issues. Additionally, horse owners have shown concerns about iron in their horse's diets. In a search of various social media platforms using the word "iron", 40 "hits" were found in the Facebook group "Equine Nutrition Education" (https://www.facebook.com/groups/equinenutritioneducation) while 50+ results were found in an online forum called the Chronicle of the Horse (https://forum.chronofhorse.com/search?q=iron).

Captive black rhinoceros are subject to iron overload (i.e., an accumulation of excess iron) (Paglia & Dennis, 1999) and share similarities with horses as they belong in the same order, Perissodactyla, and are also hindgut fermenters. Thus, horses were utilized as a model animal to assess iron status and insulin responses (Nielsen et al., 2012). When horses were fed dextrose and corn, higher ferritin concentrations were seen in horses with a higher insulin response, suggesting a potential link between iron stores and insulin resistance (Nielsen et al., 2012). Another research group has also shown a correlation between iron overload and insulin resistance (Kellon & Gustafson, 2019). Kellon and Gustafson found elevated serum ferritin concentrations in hyperinsulinemia horses (n=33), with 100% of these concentrations being above the published reference range (43-261 ng/ml (Smith et al., 1984)) (Kellon & Gustafson,

2019), however a flaw of this study was that they only utilized hyperinsulinemic horses and no controls. Based on both studies, it suggests that body iron stores (and subsequent elevated serum ferritin) and insulin resistance may be interrelated.

Despite these studies finding this potential correlation, there is a recent study that did not see any correlation between dietary iron and insulin resistance (IR) in horses. A survey-based study of trainers was conducted to determine iron intake and incidences of IR in racing Thoroughbreds based in the United States. Racehorses were fed a daily average of 3900 mg of iron from hay and grain alone, which exceeds the current NRC requirements (NRC, 2007). Additionally, 40% of trainers utilized both a vitamin-mineral and iron-specific supplement. When respondents were asked why they supplemented iron, the main reasons for this were to prevent anemia or to boost red blood cells to increase oxygen production and subsequent performance. With the supplements, this provided an average of 500 mg of additional iron daily. Despite the high dietary intake of iron, there were no cases of IR in the survey population, however, Thoroughbreds are typically not prone to insulin resistance (McLean et al., 2022). Additionally, Pratt-Phillips et al. (2010) found that breed had a significant effect on insulin and leptin levels, in which insulin levels were typically lower in Thoroughbreds versus ponies (Pratt-Phillips et al., 2010). Therefore, it appears that breed and other factors can play a role on the propensity of insulin resistance. Although the above study did conclude that dietary iron is unlikely to be a causative factor of IR, it did have several limitations. First, racing Thoroughbreds are typically at little risk of insulin resistance due to actively being in performance conditioning. Second, survey responses may have been inaccurate if respondents did not correctly identify the type of hay or other feed as well as daily intake, thus producing inaccurate results. Third, daily iron intake was estimated from roughage values listed on Equi-

Analytical (Ithaca, NY) as individual hay analysis was not possible, therefore the daily iron value could be inaccurate. Finally, serum ferritin, a well-known indicator of body iron stores, was not evaluated as well as other parameters (serum iron, insulin, etc.), limiting the ability to accurately assess the actual iron and glucose/insulin states of this particular population. Thus, the results of the study may not necessarily be indicative of the potential correlation between iron and metabolic issues across all equine populations, especially in populations in which metabolic issues are more prevalent, such as ponies.

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# PART III: PERFORMANCE ANALYSIS

## 1.15. Introduction

Performance analysis in sports has been utilized in a plethora of human sports including football, rugby, hockey and others. This is a widely used technique which assesses key factors to provide objective feedback in order to enhance performance within a sport (McGarry et al., 2013). For performance analysis to be successful, it needs to be used concurrently by athletes, coaches and a performance analyst. With the information gained from the analysis, skill development, training plans and competition strategies can be improved and aligned to key performance indicators of the particular sport (Hughes & Bartlett, 2002; McGarry, 2009; Nicholls et al., 2019). Once a goal has been defined, performance analyst(s) will describe the performance of an athlete(s) by identifying correlations between actions (e.g., sport specific behavior) and outcomes (e.g., key performance indicators) while also considering outside factors (e.g., environmental, other competitors). By completing this process strategies to improve performance can be developed and implemented (Marlin & Williams, 2020; Williams, 2013).

Oftentimes, performance analysis is completed via video. Video-based performance analysis has been proven to be practically effective, however the impact on it is unknown due to limited research within this area (Groom et al., 2011; Nicholls et al., 2019). Despite limited research, previous studies have found that video helped with recall, developed a deeper understanding of the sport and ultimately improved player confidence (Francis & Jones, 2014; Groom & Cushion, 2004). Additionally, when assessing minors using video analysis in amateur hockey, this yielded a positive result for both coaches and athletes, with coaches noting that this type of analysis can be used as a development tool to help younger athletes get to the next level

of the sport (Lee, 2011). Thus, it does appear that video-based performance analysis can be beneficial to coaches and athletes in order to improve performance.

Despite the positive results seen in other sports, performance analysis research is limited in equestrian sports, specifically show jumping. The rest of this review will discuss the potential benefits of utilizing performance analysis in equestrian sports as well as the previous research done.

### 1.16. Performance Analysis in Equestrian Sports

Show jumping is an internationally recognized and popular equestrian sport and is a part of the equestrian sports at the Olympics. The main objective is for a rider and horse combination to complete a course of various types of jumping obstacles (e.g., verticals, oxers, liverpools) within a specific time or the fastest time without accruing any penalties, commonly referred to as faults. Faults include knock-downs, refusals, falls and going over the allotted time. Jumping obstacles can vary in type, difficulty and height depending upon the level of competition, however heights typically range from 0.70 to 1.60 meters ((USEF), 2022).

The welfare of horse and rider has become prioritized more recently, thus increasing the demand to utilize evidence-based practices across equestrian sports like show jumping (Campbell, 2021; Mills et al., 2005) through a technique like performance analysis. Through the use of this technique, performance analysis could provide strategies for riders and coaches to implement evidence-based practices when training and preparing for competitions while keeping equine welfare in mind (Marlin & Williams, 2020; Waran & Randle, 2017). However, performance analysis within equestrian sports can be complex as it mainly focuses on subjective assessments like ‘feel’ or observations (Ely et al., 2010; Williams, 2013). Additionally, subjective assessments like performance observations and ‘feel’ rely heavily on memory recall

and an individual's perception of performance, thus being subject to potential bias. Furthermore, analyzing equestrian sports requires focusing on multiple areas: the horse's performance (which can be influenced by the rider), the rider's performance (which can be influenced by the horse), the 'performance' as a whole entity and the partnership between both horse and rider (Williams, 2015). This complex partnership is compounded further as most athletes in equestrian sports often train individually and/or in isolation, meaning self-analysis and reflection is more common versus other sports, potentially leading to recall issues and/or bias. Despite the complexities of the horse and rider partnership, this dynamic can also be seen in human sports, in which performance analysis has been shown to be successful (Francis & Jones, 2014; Groom & Cushion, 2004; Lee, 2011). Therefore, performance analysis should be implemented within equestrian sports to collect data so riders, trainers and coaches can utilize strategies for training and competition to enhance performance.

Although there are limited studies using performance analysis within the equestrian sport of show jumping, a few studies have yielded promising results to support the implementation of this technique more frequently. Eight 5* Federation Equestre International (FEI) competitions across Europe were analyzed via video and notational analysis by Marlin and Williams (2020) to assess if there were relationships between faults and type of fence, approach and direction (Duthie et al., 2003; Marlin & Williams, 2020). The most common type of faults were knock-downs at 5.5% while the least common was refusals at 0.2%. When analyzing fault location, knock-downs were 2.8 times more likely to occur within the second half of the course versus the first ($P<0.05$) (Marlin & Williams, 2020). Despite faults being distributed across fence types, faults were more likely to occur at upright fences and jumping obstacles within a combination. Based on the results of this study, there are evident patterns in how faults accrue. Additionally,

by analyzing performance through notational analysis, this could be a way to improve competition strategies in order to reduce the possibility of faults and thus enhance performance (Marlin & Williams, 2020).

Ničová & Bartošová (2022) analyzed the 1st and 2nd rounds of 5* FEI show jumping competitions (Ničová & Bartošová, 2022). Faults within the 1st round were more likely to occur at an upright fence with water and in both the 1st and 2nd round, faults were more likely to occur at fences within a combination versus single obstacles. Additionally, the probability of faults decreased in the 2nd round (e.g., the jump off) with a higher speed ($P<0.01$) (Ničová & Bartošová, 2022). The results of this study are in agreement with Marlin and Williams, in which there are clear patterns of fault accrual in elite levels of show jumping. Therefore, this useful information can be used by riders and trainers to prepare for future competitions.

Despite the limited studies done assessing elite level competitions, there has been even more limited research done evaluating performance at lower-level show jumping competitions. Data was collected from regional competitions with jump heights ranging from 1.0-1.4 meters. Similar to previous studies assessing elite level competitions, upright fences were more likely to be knocked down (Marlin & Williams, 2020; Ničová & Bartošová, 2022), but this study also found oxers (e.g., a spread obstacle) to be most frequently knocked down. Additionally, faults were more often seen within combinations compared to singular fences. This study also assessed color and the occurrence of faults, finding that most knock-downs occurred at white, brown-red, green-blue and blue-red fences (Stachurska et al., 2002). However, a more recent study analyzing elite show jumping did not find any correlation between fault accrual and fence color combinations but did see a correlation between faults and fence design (Williams et al., 2022).

Although further research needs to be done within the area of performance analysis in equestrian sports like show jumping, the research done in previous studies as well as other sports showcases the benefits to utilizing this methodology in order to improve training and competition strategies and ultimately enhancing performance.

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# CHAPTER 2: STUDY 1

## Feral equine populations have different fecal microbiome and *in-vitro* fermentation patterns than domesticated populations

### 2.1. Abstract

The equine hindgut possesses an important microbial community, in which microbes ferment fibrous materials and produce volatile fatty acids, which are able to be utilized to meet energy needs. To date, the fecal microbiome and fermentation patterns within a feral population of horses in the United States has not been studied. Thus, the purpose of this study was to evaluate the fecal microbiome and fermentation patterns between a feral and two domesticated populations. Fecal samples were taken from three populations: a feral population of horses located on the Shackleford Banks in the Outer Banks of North Carolina and two domesticated populations: 1) research and teaching horses at the NCSU Equine Educational Unit and 2) privately owned horses. Horses were monitored and samples were collected immediately following a void by swabbing the middle of the void and collecting fecal balls. Samples for microbiome analysis were sent to Purina Animal Nutrition (Gray Summit, MO) to sequence the 16S rRNA gene and statistical analysis was performed in R Studio (Version 4.1.1). For the in-vitro fermentation model, fecal samples were immediately placed on dry ice after collection and frozen at -80°C until analysis. Individual samples were pooled to form a representative sample for each population, an inoculum was formed and added to Wheaton bottles containing an alfalfa (A) or alfalfa starch (AS) treatment. Bottles were run in triplicate (n=72) and incubated for 0, 2, 4 and 24 hours. After each respective incubation period, pH, methane and volatile fatty acids (VFA) were measured. Statistical analysis was performed in SAS Studio (SAS Institute Inc., Cary, NC) and a P-value of $\leq 0.05$ was considered significant. The most predominant phyla

within the study were Firmicutes and Bacteroidetes, with differential abundance tests revealing Firmicutes was more enriched within the feral population while Bacteroidetes was more enriched within the NCSU population. Furthermore, differentially abundant taxa at the Order level included Spirochaetales and Clostridiales within the Shackleford population and Erysipelotrichales and Bacteroidales within the privately owned and NCSU populations, respectively. Bray Curtis revealed a clear, visual separation between the feral (e.g., Shackleford) and domesticated (e.g., NCSU, privately owned) populations and this was significant with a PERMANOVA test ($P=0.01$). For the in-vitro fermentation, acetate was the highest produced VFA, with concentrations being highest within the privately owned population, especially in the AS treatment. When considering propionate, there was a population x time interaction ($P<0.0001$). Butyrate was highest within the feral population ($P<0.0001$) with a concentration of 16.4 molar % at 24 hours for the A treatment. Based on the results, the microbiome of the feral horses was more diverse while fermentation patterns reflected the microbial composition. Future studies should continue to focus on how dietary and other management practices impact the equine microbial community and subsequent VFA production.

## 2.2. Introduction

Horses are hindgut fermenters that possess an enlarged cecum and colon, collectively called the hindgut. The hindgut is an important component within the digestive tract, comprising about 61% of the total volume, and is where dietary fibrous materials consumed is broken down by microbes (Geor et al., 2013). Fermentation by resident hindgut microbiota generate end-products including methane and volatile fatty acids (VFAs) such as acetate, propionate and butyrate. VFAs can be utilized for energy production, with Glinsky et al. (1976) estimating that these can provide up to 30% of a horse's maintenance energy needs (Glinsky et al., 1976) while Vermorel

et al. (1997) found that 80% of energy needs can be met from VFAs when consuming a primarily hay diet (Vermorel et al., 1997). The main volatile fatty acid produced within the large intestine is acetate, which can be utilized to form acetyl-CoA and enter directly into the Krebs cycle (NRC, 2007). Butyrate is thought to be taken up by colonocytes within the colon to serve as a fuel source to support the intestinal epithelium (Siddiqui & Cresci, 2021), regulate energy metabolism and enhance insulin sensitivity (Magne et al., 2020). Propionate can also be utilized as a fuel source by synthesizing glucose in the liver via gluconeogenesis (Den Besten et al., 2013). Additionally, microbes synthesize the water-soluble B vitamins and fat-soluble Vitamin K such that these do not necessarily have to be provided in a horse's ration (Geor et al., 2013).

Comparing the microbial population between different equine populations can be beneficial to understand how dietary and management practices implemented in domesticated horses can affect the composition of the microbiota. In a recent study by Ang et al. (2022) who examined feral and domesticated horses in South America, Asia and Europe, it was found that feral horses possessed a higher abundance of eukaryotes and viruses while domesticated horses' microbiomes contained genes enriched in tetracycline resistance, reflecting the use of antibiotics in management practices. Additionally, enzymes encoding for carbohydrate metabolism were higher in feral versus domesticated horses, indicating feral animals are more readily able to catabolize complex carbohydrates due to a more varied diet (Ang et al., 2022). Przewalski's horses (PH) were found to have a more diverse composition compared to domesticated populations and this was driven by different ratios of taxa in orders such as Clostridiales, Spirochaetales and Bacteroidales. Life history also influenced the microbiome, in which the population of PH born in captivity had a lower microbial diversity compared to PH populations born on natural reserves (Metcalf et al., 2017). Thus, the diversity seen within Przewalski's

horses compared to domesticated horses could be driven by life history as well as more variations within the diet. The level of domestication may also influence the fecal microbiome composition. In the United Kingdom, feral (e.g., low management) Exmoor ponies had higher abundances of Methanobacteria while domesticated (e.g., high management) Exmoor ponies possessed higher abundances from the phyla Proteobacteria and Tenericutes. Finally, the semi-feral (e.g., medium management) population had intermediate levels of these taxa as well as the highest within-group variation, indicating management levels influence the fecal microbial composition (Bull et al., 2024).

In-vitro fermentation models can be beneficial to assess the amounts of gases and VFAs produced, thus quantifying fermentation occurring within the hindgut. Dietary composition and its influence on VFAs have been studied extensively, with several studies finding that a high versus low starch diet changes the percentage of volatile fatty acids produced (Hintz et al., 1971; Julliand et al., 2001; Swyers et al., 2008). In a study by Hansen et al. (2015), a diet with a more rapid and higher nutrient availability (e.g., oats) resulted in lower ammonia and increased VFA levels, in particular propionate, versus horses fed a slower and low nutrient availability (e.g., hay) (Hansen et al., 2015). In a recent study, Gandarillas et al. (2021) found that a higher forage diet produced a greater acetate to propionate ratio (Gandarillas et al., 2021). When assessing cecal fluid and the composition of diet on VFA production, an increase in fiber and starch content resulted in an increase in total VFA production, especially propionate (Brøkner et al., 2016; Jensen et al., 2010).

Within the United States, Schoenecker et al. (2021) estimated there are 300,000 feral horses while the American Horse Council estimated there are 6.5 million domesticated horses (Council, 2023; Schoenecker et al., 2021). To the author's knowledge, the fecal microbiome and

fermentation patterns has not been analyzed in feral populations of horses residing within the United States. Therefore, the purpose of this study was to assess the fecal microbiome and in-vitro fermentation within a feral equine population and two domesticated populations with varying habitual diets. It was hypothesized there would be a higher microbial diversity within the feral population compared to the domesticated horses and that the fermentation patterns would be similar to the habitual diet of the respective populations.

## 2.3. Materials and Methods

### *2.3.1. Sample Collection*

Fecal samples were collected from three different groups of horses, one of which was a feral population while the other two were domesticated. The first group were feral horses living on the Shackleford Banks (denoted as Shackleford; n=24), which is the southernmost barrier island of the Cape Lookout National Seashore on the Outer Banks in North Carolina, USA (Coordinates: 34.68175081700982, -76.63283342061129). There are approximately 100 wild horses living on this island in harems and are thought to be of Spanish ancestry (NPS). Due to the desolate nature of this island, these horses consume only natural food sources present consisting of *Spartina* marsh and island grass as well as *Uniola* (sea oats) (NPS). Horses are minimally managed, although immunocontraception may be utilized to control population growth when needed.

The second and third group of horses were domesticated populations. The second group were research and teaching horses housed at the North Carolina State University Equine Educational Unit (denoted as NCSU; n=18) in Raleigh, North Carolina, USA (Coordinates: 35.82100946237566, -78.72135188040144). The typical diet of NCSU horses consisted of cool season mixed pastures although horses were supplemented with hay and concentrates when warranted. Additionally, horses had access to free choice salt blocks. Finally, the last group of

horses were privately owned horses (denoted as Privately Owned; n=36) located in Durham, North Carolina, USA (Coordinates: 36.00375814284245, -78.74401786335955), in which the typical diet consisted of hay, concentrates and minimal pasture. Additionally, these horses were in moderate work as lesson and show horses.

Due to being unable to get within 50 feet of the Shackleford horses in order to comply with National Park Service rules, the following sample collection method was utilized throughout the study. Horses were closely monitored for a fecal void and once a void was completed, samples were collected within approximately 2 minutes. For the microbiome analysis (Purina Animal Nutrition Emerging Technology Center, Gray Summit, MO), a fecal swab was used to swab the middle of the void and then was placed into a sample container tube containing 500 uL of DNA/RNA shield (Zymo Research, Irvine, CA). For the in-vitro fermentation analysis, approximately 100 grams (g) of fecal balls were taken from the void, placed into a Ziploc freezer bag and then immediately placed on dry ice. Upon arrival at the North Carolina State University lab, samples were frozen at -80°C until analysis. One sample for the microbiome analysis and one sample for the in-vitro fermentation was collected per horse across the three populations.

North Carolina State University's IACUC approval was sought and the study was deemed exempt. A National Park Service Permit (Application #686325) was submitted and obtained from the Park Research Coordinator at Cape Lookout National Seashore in order to collect fecal samples at the Shackleford Banks.

*2.3.2. Microbiome Analysis – Bacterial DNA Extraction*

Following collection, all samples were sent to the Purina Animal Nutrition Emerging Technology Center (Gray Summit, MO) and stored at -80°C until analysis. Bacterial DNA was extracted using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep DNA extraction

kit (Zymo Research, Irvine, CA) according to the kit protocol with the exclusion of the BashingBead™ buffer due to the use of DNA/RNA Shield as a DNA preservation medium. This was done to avoid the formation of a precipitate that would impact DNA quality and downstream analysis. Instead, 300 µL of DNA/RNA Shield™ was added to each sample, bringing the total volume up to 800 µL. The lysis step was then carried out within each sample tube, including the swab, and the remainder of the protocol was followed accordingly. Sample quality was assessed using spectrophotometry (Nanodrop Lite™, Thermo Fisher Scientific, Waltham MA) and was defined as an absorbance (260/280 nm) ratio of approximately 1.8.

*2.3.3. Microbiome Analysis – 16S Library Preparation and Sequencing*

Library preparation was carried out according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego CA). Samples were diluted to 5 ng/µL into 96-well plates (Bio-Rad Laboratories, Hercules, CA) which contained 1 negative control of PCR grade water and 95 samples. PCR amplification steps were conducted using the T100™ Thermo Cycler (Bio-Rad, Hercules, CA). The V3 and V4 hypervariable regions of the 16S rRNA gene were amplified through PCR using the 341F and 805R primer pair as described by (Klindworth et al., 2013) and contained the following sequences. The resulting amplicon length was approximately 460 base pairs (bp).

Forward (341F; 5'-3'):

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse (805R; 5'-3'):

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Following amplicon PCR, samples were cleaned using AMPure XP beads (Beckman Coulter, Pasadena, CA). Amplicon length was then assessed using gel electrophoresis. Illumina IDT Unique Dual (UD) index primers (set A) were used during index PCR to create the genomic libraries. Libraries were cleaned with the AMPure XP beads and stored at -20°C for up to a week prior to sequencing. Prior to sequencing, libraries were quantified in triplicate using fluorometric quantification (Qubit: 1x ds HS Assay Kit, Thermo Fisher Scientific, Waltham MA) and all libraries were normalized to a concentration of 4 nM using 10 mM Tris-HCl (pH 8.5) and pooled. All samples from the current study were combined with samples from several unrelated projects on a single sequencing run containing a total of 285 biological samples, 3 negative controls, and 1 positive control (ZymoBIOMICS™ Microbial Community Standard; Zymo Research). Samples were sequenced on the Illumina MiSeq sequencer using the Illumina MiSeq Reagent Kit v3. Sample data were demultiplexed and FASTQ files were generated using the Local Run Manager software on the sequencer.

*2.3.4. Microbiome Analysis – Bioinformatics and Statistical Analysis*

FASTQ files were exported to the Purina MQ™ Bioinformatics platform using paired-end reads. Data were filtered, processed and trimmed through DADA2 (Callahan et al., 2016) using the QIIME2 pipeline (Bolyen et al., 2019). Trimming parameters were set to maintain quality (Q >30) as indicated by the interactive quality plot provided by the q2-demux plugin (Callahan et al., 2016). Amplicon sequence variants (ASVs) were aligned and used to generate a phylogenetic tree using the mafft (Katoh et al., 2002) and fasttree (Price et al., 2010) plugins, respectively. To eliminate samples with a low sampling depth, it was determined that >20,085 ASVs would be the cutoff. Taxonomic classification was performed on the representative ASVs with the q2-feature-classifier plugin using a taxonomic classifier based on the SILVA (Release 132) genomic

database (Bokulich et al., 2018). The classifier was trained to the V3 and V4 hypervariable regions using the classify-sklearn plugin (McDonald et al., 2012). Diversity analysis was conducted using the q2-diversity plugin (Faith, 1992).

Statistical analysis was performed in R Version 4.1.1 (Boston, MA) using the microbiome package (Lahti & Shetty, 2018). Relative abundance by population thresholds were determined with the detection set at 1% and prevalence at 70%, meaning that a microbe must be present in at least 1% abundance across 70% of all samples. Linear discriminant analysis effect size (LEfSe) was used to identify differentially abundant taxa and generate a LDA score to estimate the effect size of each differentially abundant feature (Fisher, 1936; Segata et al., 2011). Alpha diversity metrics utilized to assess the diversity within individual samples were Observed (Richness), Shannon Index (Weaver, 1963) and Simpson Index (Simpson, 1949). A Kruskal-Wallis rank sum test was used to determine if there was a significant difference between the three populations (Kruskal & Wallis, 1952). Beta diversity metrics to assess diversity amongst the difference horse populations were tested using: Bray-Curtis (Beals, 1984) and Weighted UniFrac (Lozupone & Knight, 2005) and principal coordinate analysis (PCoA) plots were generated (Gower, 1966). A PERMANOVA (Adonis) test and pairwise comparisons were used to determine differences between the three populations (Anderson, 2008). Statistical significance was set at $P \leq 0.05$.

*2.3.5. In-Vitro Fermentation Analysis*

The in-vitro fermentation model was conducted according to the procedure outlined by Goering and van Soest (Goering & Van Soest, 1970) with modifications as deemed necessary. Two different treatment substrates were used: alfalfa (denoted as A) and alfalfa plus starch (denoted as AS). The starch used for the AS treatment was cornstarch (S4126, Millipore Sigma,

Burlington, MA). For the A treatment, approximately 1 g of alfalfa was weighed out while approximately 0.8 g of alfalfa and 0.2 g of starch was used for the AS treatment. Once the substrates were weighed out, they were added to glass, 125 milliliter (ml) Wheaton bottles (DWK Life Sciences, Millville, NJ) with a septum screw cap.

Fresh fecal samples for the in-vitro fermentation procedure were unable to be used due to geographic constraints so frozen samples were utilized. Frozen fecal inocula has been successfully used in previous studies in swine (Pastorelli et al., 2014) and humans (de Carvalho et al., 2021; Pérez-Burillo et al., 2021) without significantly affecting microbial stability and fermentation. Individual horse fecal samples were thawed, pooled together and mixed thoroughly to form a representative sample for each equine population: Shackleford, NCSU and Privately Owned in order to obtain at least 400 g of fecal material per population. Once the representative samples were formed and mixed, 100 g of fecal material and 200 ml of anaerobic medium buffer were blended for 15 seconds in a Waring blender. After blending was completed, the mix was filtered through a double-layer cheese cloth and 30 ml of fecal inoculum were pipetted into the Wheaton bottles with the treatment substrates. All bottles were purged with $CO_2$, capped and placed in a 39°C water bath for an incubation period of either 0, 2, 4 and 24 hours. Bottles (n=72) were run in triplicate for population, treatment substrate and incubation period. Blank bottles (n=6) were also run but were not included in statistical analysis. In total, 78 bottles were run.

Once the incubation period was complete (0, 2, 4 or 24 hours), bottles were removed from the water bath and placed on ice to stop microbial fermentation. Gas samples (10 μL) were taken prior to opening the bottle via a gastight syringe (Hamilton Co., Reno, NV). Methane gas concentrations were measured in nanomoles per milliliter (nmol/ml) via gas-liquid

chromatography (GLC) (model CP-380 Gas Chromatograph, Varian, Walnut Creek, CA) equipped with a Molsieve 5A 45/60 mesh stainless steel column (Supelco Inc., Bellefonte, PA). The pH of the fecal inoculum was measured using a pH probe once methane was measured. Finally, VFA analysis was completed by mixing 1 mL of inoculum with 0.2 mL of metaphosphoric acid and an internal standard (2-ethylbutyric acid). This was centrifuged (model Micromax, International Equipment Co., Needham Heights, MA) at 21,000 x g for 10 min at 4°C and then analyzed via GLC (model CP-3380 Gas Chromatograph, Varian, Walnut Creek, CA) using a fused-silica capillary column, 30 m x 0.25 mm with 0.25-μm film thickness (Nukol, Supelco Inc., Bellefonte, PA) according to methods by Fellner et al. (1997) (Fellner et al., 1997). Volatile fatty acids, specifically acetate, propionate, butyrate and isoacids (e.g., isobutyric and isovaleric) were measured in molar percentage (mol/100 mol). Additionally, total VFAs (e.g., acetate, propionate, butyrate, isobutyrate, isovalerate, valerate) were measured in mM.

*2.3.6. In-Vitro Fermentation Statistical Analysis*

All statistical analyses were performed in SAS Studio (Version 3.81, SAS Institute, Inc., Cary, NC) using the PROC MIXED procedure. The effects of population (Shackleford, NCSU and Privately Owned), treatment (A, AS) and time (0, 2, 4, 24 hours) were performed on all variables measured (pH, methane, acetate, propionate, butyrate, isoacids and total VFAs). LS MEANS was used to compute the effect of each variable and a post-hoc Tukey's HSD test was performed. In addition, the PROC MIXED procedure was run on all variables by time, in which the effects of population and treatment were assessed at each time period (0, 2, 4, 24 hours). Summary statistics (mean) were also generated in SAS Studio. Statistical significance was set at $P \leq 0.05$.

## 2.4. Results

### *2.4.1. Microbiome Results*

After removing samples with a low sampling depth (>20,085 ASVs), 78 samples were analyzed across the three populations (Shackleford: n=24; NCSU: n=18; privately owned: n=36). The top phyla observed within the study included Firmicutes, Bacteroidetes, other, Spirochaetes, Kiritimatiellaeota, Fibrobacteres and Tenericutes. Firmicutes was the most predominant phylum at 49% abundance within the feral population of horses (e.g., Shackleford) while Bacteroidetes was the 2$^{nd}$ most predominant phylum at 36% abundance within both domesticated populations (e.g., NCSU, privately owned) (Figure 2.1). Phyla designated under "other" included ones that did not meet the relative abundance thresholds (i.e., 1% detection, 70% relevance). Spirochaetes was more abundant within the Shackleford population at 5% versus the two domesticated populations.

Differences in abundance of specific taxa between the three study populations were evaluated using LEfSe analysis (Segata et al., 2011) and results are presented visually in Figure 2.2. The phyla Firmicutes and Spirochaetes were found to be more enriched in the Shackleford population while Bacteroidetes was more enriched in one of the domesticated populations (e.g., NCSU) (Figure 2.2). When considering class, Clostridia and Spirochaetia were more enriched in the feral population whereas Bacteroidia and Erysipelotrichia was more abundant within the NCSU and privately owned population, respectively. When looking at order, differentially abundant taxa included Clostridiales and Spirochaetales within the feral Shackleford population, Erysipelotrichales within the privately owned population and Bacteroidales within the NCSU population (Figure 2.2). Finally, when assessing family, *Lachnospiraceae* and *Clostridiaceae*

were enriched within the Shackleford population while *Erysipelotrichaceae* and *Prevotellaceae* were enriched in the privately owned and NCSU population, respectively.

Alpha diversity metrics assessed included Observed (Richness), Shannon Index and Simpson Index. A Kruskal-Wallis rank sum test revealed differences among the three groups for Observed (Richness) and Shannon Index ($P=0.05$) but not Simpson Index ($P>0.05$) (Figure 2.3). When looking at Shannon Index specifically, pairwise comparisons found significant differences between the NCSU and Shackleford population ($P=0.03$), NCSU and privately owned population ($P=0.005$) but not the Shackleford and privately owned population ($P=0.3$) (Figure 2.4).

Beta diversity metrics utilized included Bray Curtis and Weighted UniFrac and the associated principal coordinate analysis (PCoA) plots are presented in Figure 2.5 and 2.6. On the Bray Curtis PCoA plot, there is a clear, visual separation between the two domesticated populations (e.g., NCSU, privately owned) and the feral population (e.g., Shackleford) (Figure 2.5). Using a PERMANOVA test (Adonis), differences among the populations were found to be significant ($P=0.001$) as well as the homogeneity condition ($P=0.001$). Pairwise comparisons were determined to be significant and were as follows: NCSU versus privately owned ($P<0.001$), NCSU versus Shackleford ($P=0.001$), privately owned versus NCSU ($P<0.001$), privately owned versus Shackleford ($P=0.002$), Shackleford versus NCSU ($P<0.001$) and Shackleford versus privately owned ($P<0.001$). The PCoA plot for Weighted UniFrac is shown in Figure 2.6. A PERMANOVA (adonis) test also revealed significant differences ($P=0.001$) and pairwise comparisons among the three respective populations were also significantly different ($P<0.05$).

*2.4.2. In-Vitro Fermentation Results*

Mean methane and pH values are presented in Table 2.1. There was a significant population x time interaction ($P=0.01$), treatment x time interaction ($P=0.05$) as well as a

significant treatment (P=0.03) and time effect (P<0.05) for methane (Table 2.3). Additionally, at 0 hours, differences in populations were found to be significant (P=0.002) while at 24 hours, differences in treatment were found to be significant (P=0.006). Methane concentrations were highest at 0 hours across all populations with the AS treatment, however at 24 hours, methane concentrations for the AS treatment were similar to 0 hours in privately owned horses (Table 2.1). For pH, there was a significant population x time interaction (P=0.0003) as well as a treatment x time interaction (P=0.0006) (Table 2.3). pH values were relatively similar between the two treatments across all populations and time points throughout the in-vitro fermentation with pH ranging from 5.6-7.1 and 5.2-6.8 for the A and AS treatment, respectively.

Acetate values are presented in Table 2.2. There was a significant treatment and population effect (P<0.05) as well as a population x treatment x time (P<0.0001) interaction (Table 2.3). At 24 hours, there was a significant population and treatment effect (P<0.05) and population x treatment interaction (P=0.0003), in which acetate concentrations were highest in the privately owned horses with the AS treatment (Table 2.2). Additionally, acetate was the most produced VFA across both treatments, as values were higher compared to the other volatile fatty acids measured.

Propionate values are presented in Table 2.2. There was a significant population and treatment effect (P<0.05) as well as a population x time interaction (P<0.0001) (Table 2.3). There was also a significant time effect (P<0.0001) (Table 2.3), in which propionate values were highest at 0 hours versus 24 hours across all populations and treatments (Table 2.2).

Butyrate values are presented in Table 2.2. There was a population, treatment and time effect (P<0.05). Additionally, a population x treatment x time interaction was found to be significant (P<0.0001) (Table 2.3). At 24 hours, there was a population x treatment interaction

(P=0.005), in which the Shackleford population had the highest butyrate values with the A treatment while the NCSU population had the highest values with the AS treatment (Table 2.2).

Isoacids, defined as isobutyrate and isovalerate, values are presented in Table 2.2. There was a significant population and time effect (P<0.05) as well as a population x time interaction (P<0.0001) (Table 2.3). At 0 hours, isoacid values were the highest across all populations (P=0.008), in which these values were highest in the NCSU population with the A treatment and within the Shackleford population with the AS treatment (Table 2.2).

Total volatile fatty acid (VFA) values are presented in Table 2.2. There was a significant population, treatment and time effect (P<0.05). Additionally, a population x treatment x time interaction was significant (P=0.02) (Table 2.3). Total VFA production was lowest at 0 hours and increased throughout the 24 hours, in which values across both treatments and all populations were highest at 24 hours (Table 2.2). At 24 hours, there was a significant treatment effect (P=0.01), in which values for the Shackleford and NCSU populations were higher for the A treatment versus the AS treatment (Table 2.2). However, total VFA values in the privately owned population were relatively the same across the two treatments at 24 hours.

## 2.5. Discussion

### *2.5.1. Microbiome*

The fecal microbiome results revealed that the feral Shackleford horses had a more diverse microbiome when compared to the domesticated populations, with these differences among the populations possibly influenced by the habitual diet. Firmicutes and Bacteroidetes were found to be the 1$^{st}$ and 2$^{nd}$ most predominant phyla within fecal samples across the feral and domesticated populations, which is in agreement with previous studies (Bull et al., 2024; Costa et al., 2015; Costa et al., 2012; Dougal et al., 2013; Li et al., 2019). Additionally, LEfSe analysis

revealed that Firmicutes was significantly enriched within the Shackleford population. Bacteroidetes was more abundant at 36% within the domesticated populations compared to the feral population but LEfSe analysis determined that this phylum was differentially abundant within the NCSU population only. Spirochaetes was the 4$^{th}$ most predominant phylum and was found to be enriched within the Shackleford population, which is in agreement with other studies that have found this phylum to be present in smaller quantities within the equine fecal microbiome (Dougal et al., 2014; Dougal et al., 2013; Edwards et al., 2020; Morrison et al., 2018).

The classes Clostridia and Spirochaetia were differentially abundant within the feral population while differences in Bacteroidia and Erysipelotrichia were found in the NCSU and privately owned population, respectively. Clostridia, a member of the Firmicutes phylum, has been found to be more abundant within a population of Exmoor ponies that were minimally managed when compared to ponies undergoing increased levels of management using 16S rRNA sequencing (Bull et al., 2024). Additionally, features within the Clostridia class decreased when horses were fed a high starch diet (Dougal et al., 2014), indicating that dietary management practices seen in domesticated horses may influence the composition of the microbial community. Differences in order were seen between the feral and domesticated populations, specifically the orders of Spirochaetales, Clostridiales, Erysipelotrichales and Bacteroidales. These results are in agreement with a previous study utilizing 16S rRNA sequencing, in which Metcalf et al. (2017) assessed the fecal microbiome of Przewalski's horses (PH) and domestic horses and discovered that these two groups microbiome were phylogenetically distinct, driven by differences in ratios of the orders Spirochaetales, Clostridiales, Erysipelotrichales and Bacteroidales (Metcalf et al., 2017).

Differences in family between the three populations included differentially abundant features from the families *Lachnospiraceae, Clostridiaceae, Erysipelotrichaceae* and *Prevotellaceae,* in which *Lacnhospiraceae* and *Clostridiaceae* was more enriched within the feral population while *Eryspielotrichaecaeae* and *Prevotellaceae* were enriched in the domesticated populations. *Lacnhospiraceae* has been found to be differentially abundant in minimally managed Exmoor ponies (Bull et al., 2024), which is in agreement with the present study. Hansen et al. (2015) determined that the cecal abundance of *Clostridiaceae* was higher in horses fed a hay diet (e.g., low and slower nutrient availability) compared to an oat diet (e.g., high and rapid nutrient availability) while horses on the oat diet had a higher abundance of *Eryspielotrichaecaeae* (Hansen et al., 2015). Finally, *Prevotellaceae,* a member of the Bacteroidetes phyla, has been found to be positively correlated with acid detergent fiber (ADF) compositions within forages (Weinert et al., 2021).

Based on differential abundance testing, it appears that the composition of the fecal microbiota may be influenced by the habitual diet of each respective population. Firmicutes was the most abundant phylum within all populations, however it was differentially abundant within the feral Shackleford horses, who eat a diverse diet consisting of *Spartina* marsh, island grass and *Uniola* (NPS). Bacteria within the Firmicutes phylum are able to process a variety of substrates in order to produce volatile fatty acids (Huang et al., 2018) and have been shown to degrade fiber within the human gut (Sun et al., 2023). Members within this phylum, such as *Lachnospiraceae* and *Clostridiaceae*, are considered fibrolytic and cellulolytic bacteria within the equine large intestine and *Lachnospiraceae* has been found to be increased in horses on a grass-based diet (Daly et al., 2012; Daly et al., 2001; Kauter et al., 2019). Thus, it appears that the microbiome within the feral population possess a higher abundance of members within the

Firmicutes phylum in order to more efficiently extract substrates from their varied diet. Bacteroidetes was found to be more abundant within the domesticated populations, however it was only differentially abundant in the NCSU population. Members within the Bacteroidetes phylum have been shown to be primary degraders of polysaccharides and oligosaccharides like starches (Flint et al., 2012; Zafar & Saier Jr, 2021). As the NCSU and privately owned population horses have a typical diet of domesticated horses versus the feral population, microbial compositional differences account for these management differences within these populations, which has been seen in other studies comparing domesticated and feral horses (Ang et al., 2022; Bull et al., 2024).

Alpha diversity metrics that showed significant differences in diversity within each population included Observed (Richness) and Shannon Index. Previous studies that have assessed feral and domesticated equine populations have reported mixed results when it comes to alpha diversity. Bull et al. (2024) found no differences between low management, medium management and high management Exmoor ponies (Bull et al., 2024) while Metcalf et al. (2017) saw differences in the fecal microbiome of Przewalski's horses who were foals versus over 1 years old (Metcalf et al., 2017). Furthermore, Ang et al. (2022) utilized shotgun sequencing and did not find any differences between feral and domestic horses using multiple alpha diversity indices (Ang et al., 2022). Beta diversity metrics like Bray Curtis revealed a clear separation between the feral and domesticated populations in the present study. Additionally, a PERMANOVA test showed significant differences among the populations. These results are in line with other studies, in which principal coordinate analysis reported clear clustering of populations of Exmoor ponies managed at different levels (Bull et al., 2024) and Przewalski's

horses had a distinct and different microbiome when compared to domestic horses (Metcalf et al., 2017).

Despite the study yielding results indicating there were clear differences in microbial composition between feral and domesticated populations possibly driven by the habitual diet, there were limitations to the study. First, due to being unable to get within 50 feet of the Shackleford horses in order to comply with National Park Service rules, samples were collected from a fecal void rather than directly from the anus. To mitigate any environmental disturbances (e.g., dirt) that could affect microbial composition, samples were taken within 2 minutes of the void. Another limitation is that metadata was not able to be obtained for all horses such as sex, age, body condition score and complete dietary information, which have been shown to influence the microbiome (Garber et al., 2020; Kauter et al., 2019). However, for diet, it was presumed that the Shackleford population were eating a more varied diet as they consumed whatever grasses were available on the island compared to the domesticated populations, in which the NCSU population were eating mostly cool-season mixed pastures with some hay and concentrates while the privately owned horses were consuming a diet primarily of hay and concentrates with minimal pasture. Finally, fecal samples were only obtained at one time point. If fecal samples were taken multiple times (e.g., in different seasons), the microbial composition could change within each population based on the season. Regardless of the limitations, the microbiome results showed a higher diversity of the microbiome within the feral population compared to the domesticated populations, likely driven by the habitual diet.

*2.5.2. In-Vitro Fermentation*

In-vitro fermentation results indicated that there were differences in fermentation patterns between the equine populations assessed as well as the two treatments (A vs. AS) over the

incubation period, likely being driven by the habitual diet of the respective populations. There were significant population x time and treatment x time interactions for methane. Additionally, methane concentrations were highest at 0 hours across all populations, with these concentrations being higher in the AS treatment. However, the privately owned population saw relatively similar concentrations at 0 and 24 hours, these values being 12.7 and 12.5 nmol/mol, respectively. When looking at previous literature, a study assessing a yeast supplement found that methane production did not begin until 10 hours of incubation and was decreased by 85% when using one type of supplement tested, indicating that yeast could affect methanogenesis (Elghandour et al., 2016). These results differ from the present study, in which methane production was higher at the start of the incubation period, however the present study utilized a substrate containing starch only. pH values stayed relatively similar throughout the incubation, however there was a population x treatment and treatment x time interaction. Previous studies have found differences in pH based on the inocula location with the cecum having a higher pH compared to fecal samples with a high starch diet (Kujawa et al., 2020) and pH being greater in forage versus concentrate substrates (Gandarillas et al., 2021).

Acetate was the most abundant VFA produced throughout the incubation, in which there was a significant interaction between population, treatment and time. Throughout the incubation, acetate concentrations were generally highest with the AS treatment, with these values being 76.5 molar % in the privately owned population at 24 hours. Acetate concentrations in the present study align with previous literature, in which Hintz et al. (1971) found that horses on an all-roughage diet produce ~74 molar percentage acetate in cecal or colonic fluid (Geor et al., 2013; Hintz et al., 1971). In a study by Biddle et al. (2013), increases in acetate concentrations were seen over a 48-hour incubation period when utilizing a starch treatment (Biddle et al.,

2013). Thus, the present study and previous research indicate a starch treatment could influence production of specific VFAs such as acetate. Gandarillas et al. (2021) measured acetate and other VFAs to assess the associative effects of forages and concentrates and found that there were no differences in acetate between forages and concentrates, however forage fermentation did result in a greater acetate to propionate ratio (Gandarillas et al., 2021).

Propionate is an important fuel source being utilized as a substrate for gluconeogenesis within the liver (Den Besten et al., 2013) and has been shown to be produced by members within the Bacteroidetes phylum (Macfarlane & Macfarlane, 2011; Magne et al., 2020). There was a significant population effect for propionate, in which concentrations of this VFA with the A treatment were higher at 17.4 and 17.4 molar % for the Shackleford and NCSU population, respectively, when compared to the privately owned population (16.5 molar %). While acetate concentrations increased throughout the incubation, propionate concentrations were highest at 0 hours then steadily decreased over the 24-hour period. Additionally, a significant treatment effect was determined for propionate when comparing the A to AS treatment. Substrates have been shown to have an effect on propionate production, in which Kujawa et al. (2020) found that a control versus high-starch substrate had a significant effect on propionate production when utilizing an in vitro gas production technique (IVGPT) (Kujawa et al., 2020). Additionally, Hansen et al. (2015) found that propionate production was increased in horses fed an oat diet (Hansen et al., 2015). Therefore, the inclusion of starch when using an IVGPT may change the microbial population's ability to ferment this substrate and in turn produce particular VFAs.

Butyrate is thought to be an important fuel source in supporting the intestinal epithelium as well as regulating energy metabolism (Magne et al., 2020; Siddiqui & Cresci, 2021) with members of the Firmicutes phylum producing more butyrate (Fei & Zhao, 2013; Magne et al.,

2020). There was a significant three-way interaction between population, treatment and time for butyrate. Additionally, at 24 hours, the Shackleford population had the highest butyrate concentrations with the A treatment at 16.4 molar %. Other in vitro studies have yielded differing results when looking at butyrate, with Biddle et al. (2013) finding that butyrate (as well as other VFAs) increased as lactate levels dropped (Biddle et al., 2013) while Kujawa et al. (2020) found no differences in butyrate between a high-starch and control substrate. However, Kujawa et al. (2020) did find that location of the inocula (e.g., cecum, rectum) had a significant effect on butyrate (Kujawa et al., 2020).

The present study found a two-way population x time interaction for isoacids, in which these concentrations were generally highest at 0 hours across the three populations. Isoacids are important in cellulose digestion and protein synthesis (Allison & Bryant, 1963; Zhang et al., 2017), such as production of branched chain amino acids like isoleucine, valine and leucine (Flachowsky et al., 1988). However, research on the fermentation of these branched-chain volatile fatty acids is limited in hindgut fermenters like horses. In sheep, it has been found that concentrations of isoacids range from 1- 4.8 molar % in rumen fluid (Flachowsky et al., 1988), while the present study found concentrations as high as 14.2 molar %, indicating that hindgut fermenters like horses may produce more isoacids overall, however this needs to be explored further. Finally, the present study saw an increase in total VFA production over the 24-hour incubation and a population x treatment x time interaction. Previous studies have found that total VFA production was different when comparing a control versus high-starch substrate (Kujawa et al., 2020), thus the present study yielded similar results. However, Gandarillas et al. (2021) did not see any effects of total VFA production when looking at different forage, concentrate and mixed forage-concentrate substrates (Gandarillas et al., 2021).

Despite seeing some differences in fermentation patterns amongst the three populations indicating the habitual diet may be playing a role, there were some limitations to the study. Due to obtaining samples hundreds of miles away from the laboratory facility, the present study was unable to perform the in-vitro fermentation with fresh fecal inocula so frozen inocula was utilized instead. However, frozen fecal inocula has been shown to not significantly change microbial stability and fermentation in swine and human studies (de Carvalho et al., 2021; Pastorelli et al., 2014; Pérez-Burillo et al., 2021). To mitigate any potential issues with utilizing frozen fecal inocula, samples were immediately placed on dry ice following collection and then frozen at -80°C until in-vitro fermentation analysis.

**2.6. Conclusion**

Fecal microbiome results revealed a more diverse microbiome within the feral population, driven by differentially abundant features like Firmicutes, Spirochaetes, Clostridiales and others, indicating the habitual diet likely influences the microbial composition in order to efficiently extract substrates from the diet. However, other factors such as age, body condition and management practices, also likely impacted the microbial community within the populations assessed. For the in-vitro fermentation, methane was typically highest within the AS versus the A treatment across the 24-hour incubation period. Acetate was the highest volatile fatty acid produced, especially in the privately owned population while butyrate concentrations were highest at 24 hours for the alfalfa treatment within the Shackleford population. Based on the results, the fecal microbiome and in-vitro fermentation are influenced by equine population and thus likely impacted by the habitual diet, showcasing the correlations among the diet, microbial composition and fermentation products produced.

## Tables and Figures

**Figure 2.1.** Bar charts depicting the relative abundance of phyla observed in fecal samples of the three equine populations (Shackleford, NCSU and privately owned) assessed with abundance thresholds set at 1% detection and 70% presence

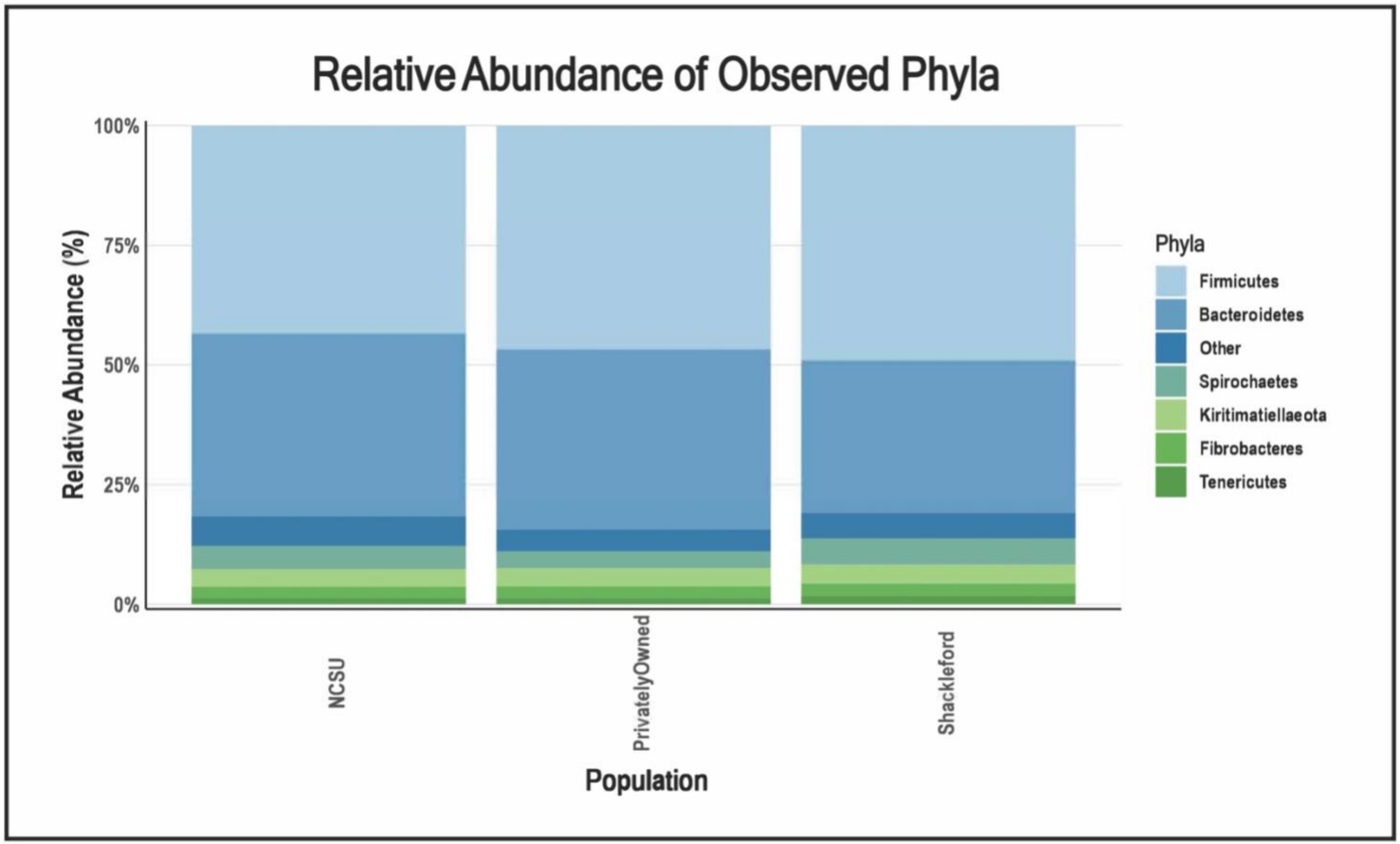

**Figure 2.2.** Bacterial taxa with a significantly different relative abundance between the three equine populations (Shackleford, NCSU, privately owned) according to Linear Discriminant Analysis Effect Size (LEfSe) analysis

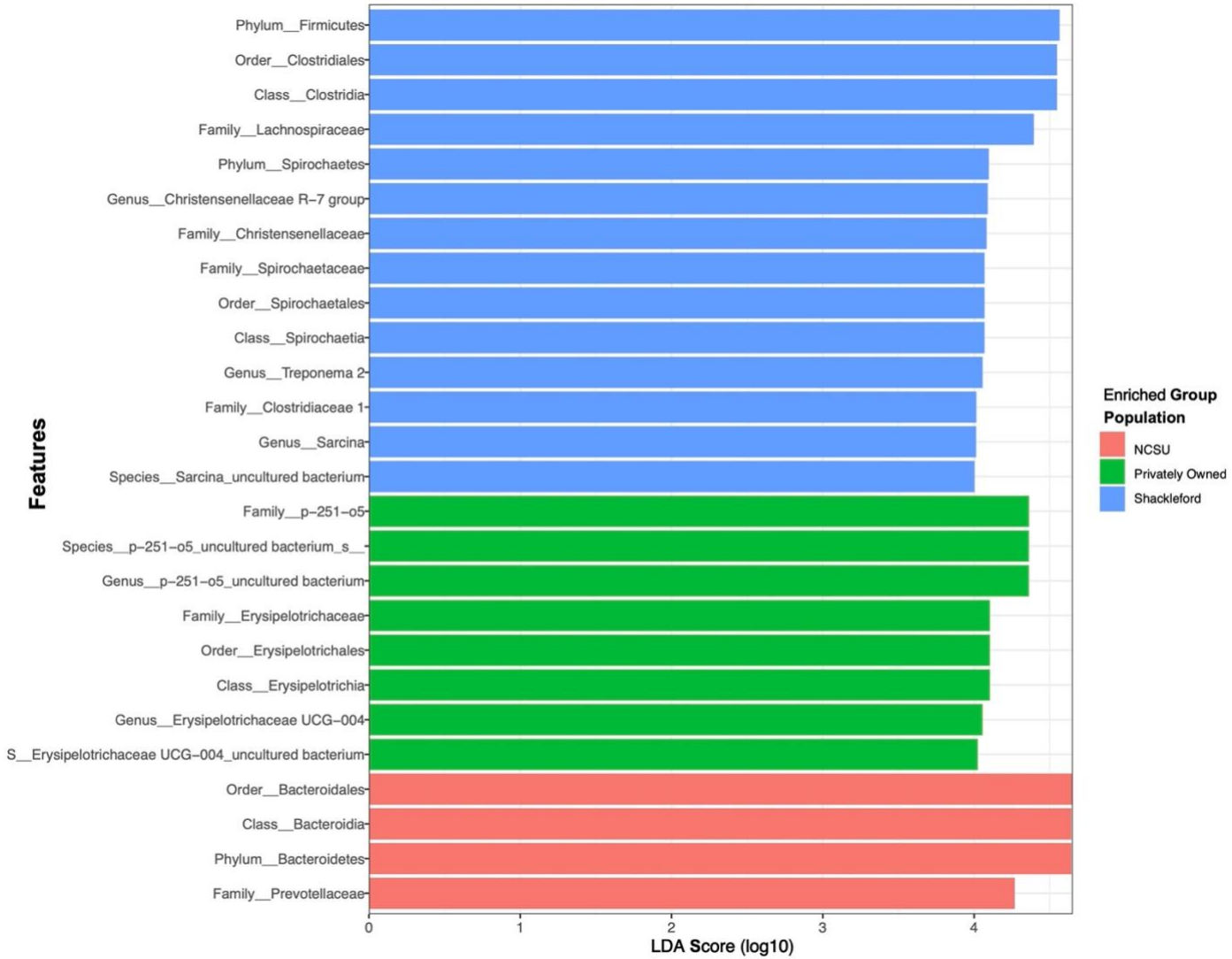

**Figure 2.3.** Boxplots depicting alpha diversity measures among the three equine populations (Shackleford, NCSU, privately owned) studied using Observed (Richness), Shannon Index and Simpson Index. Statistical significance was determined by a Kruskal-Wallis rank sum test and set at P≤0.05

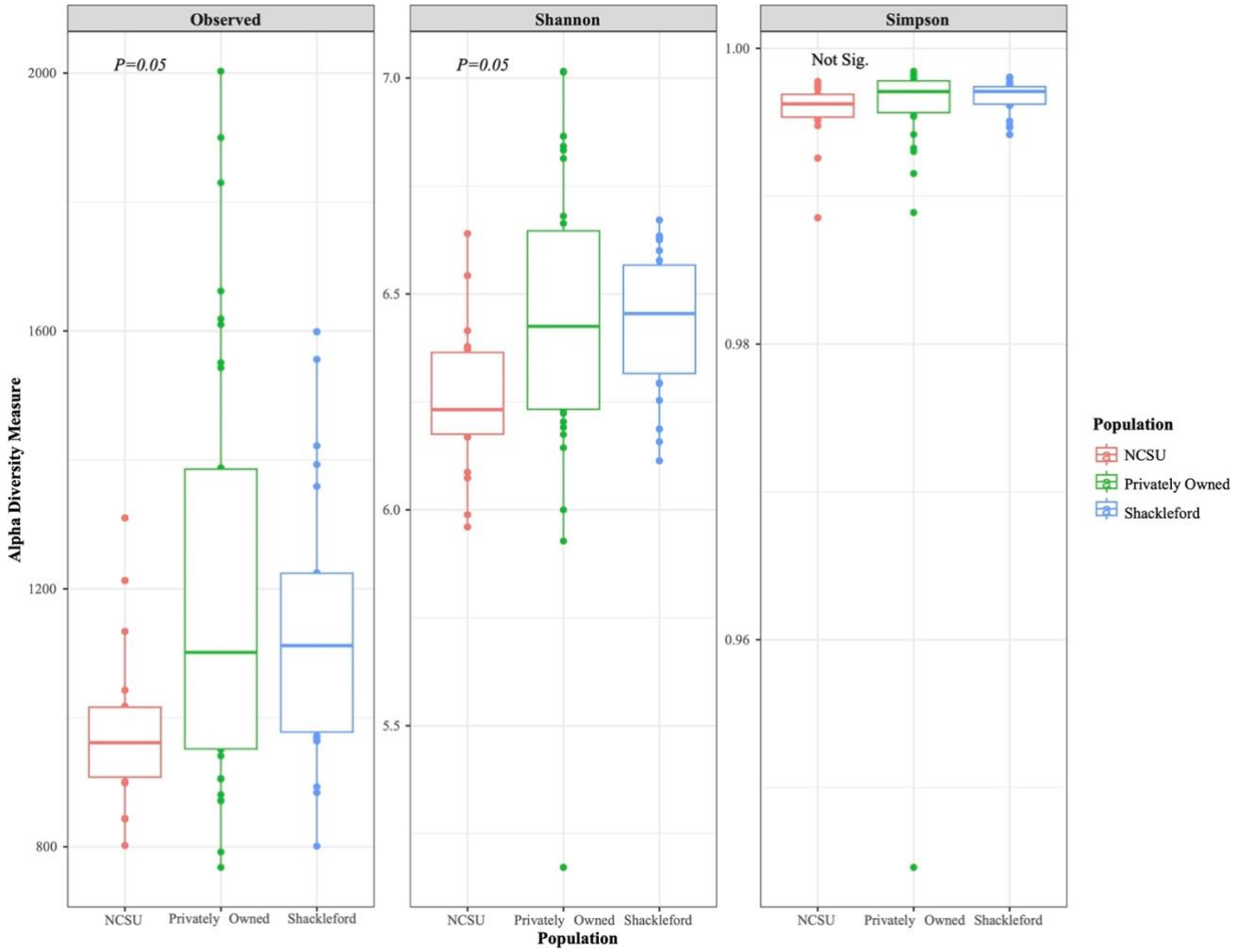

**Figure 2.4.** Boxplots depicting the alpha diversity metric Shannon Index comparing differences amongt the three populations (Shackleford, NCSU, privately owned) studied. Statistical significance was determined by a Kruskal-Wallis rank sum test and set at P≤0.05

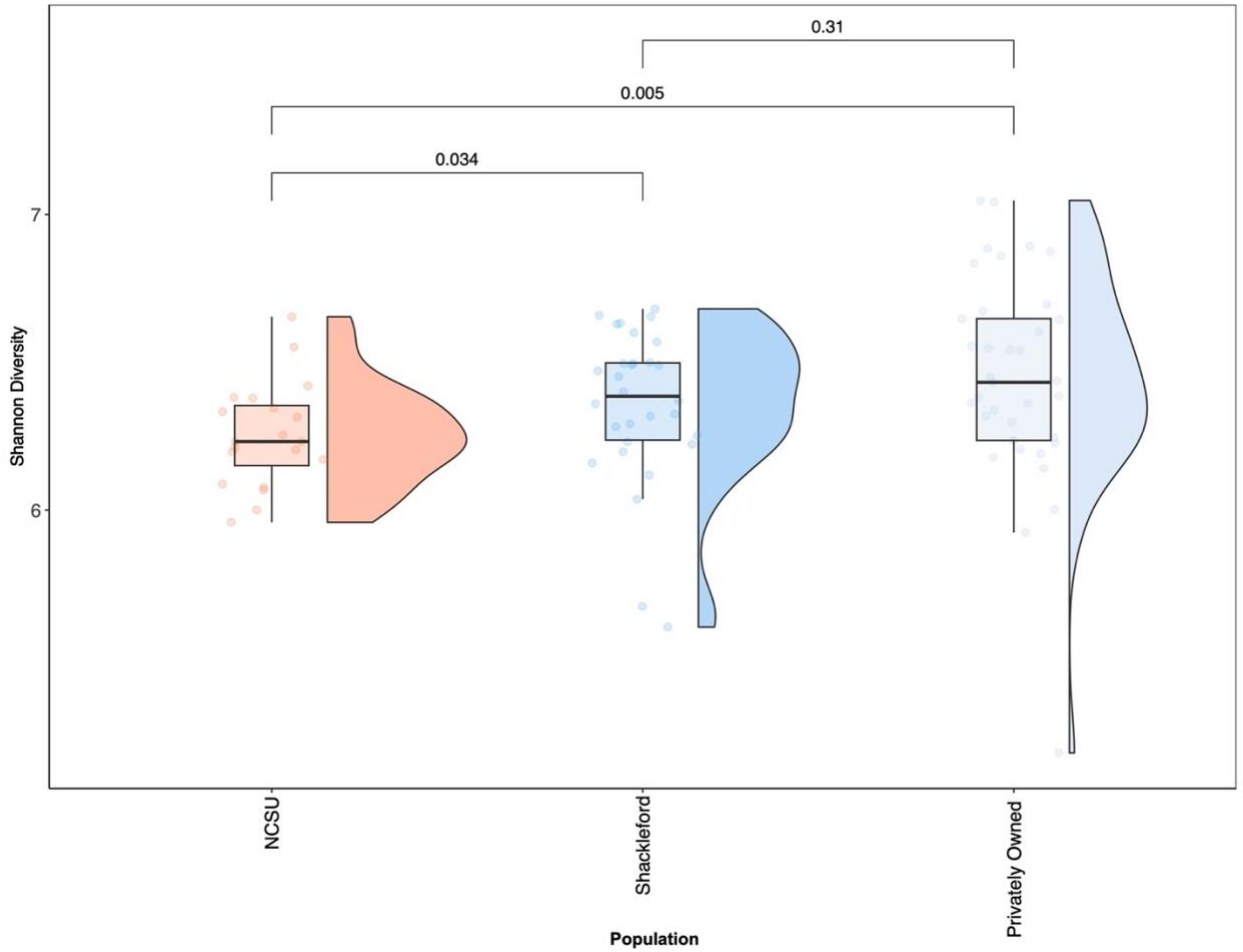

**Figure 2.5.** Principal coordinate analysis (PCoA) plot using the beta diversity metric Bray Curtis to quantify dissimilarity in species composition among the three populations (Shackleford, NCSU, privately owned) showed clear clustering between the feral (Shackleford) and domesticated populations (NCSU, privately owned). The percentage values within the brackets on Axis 1 and 2 refer to the percentage of explained variation

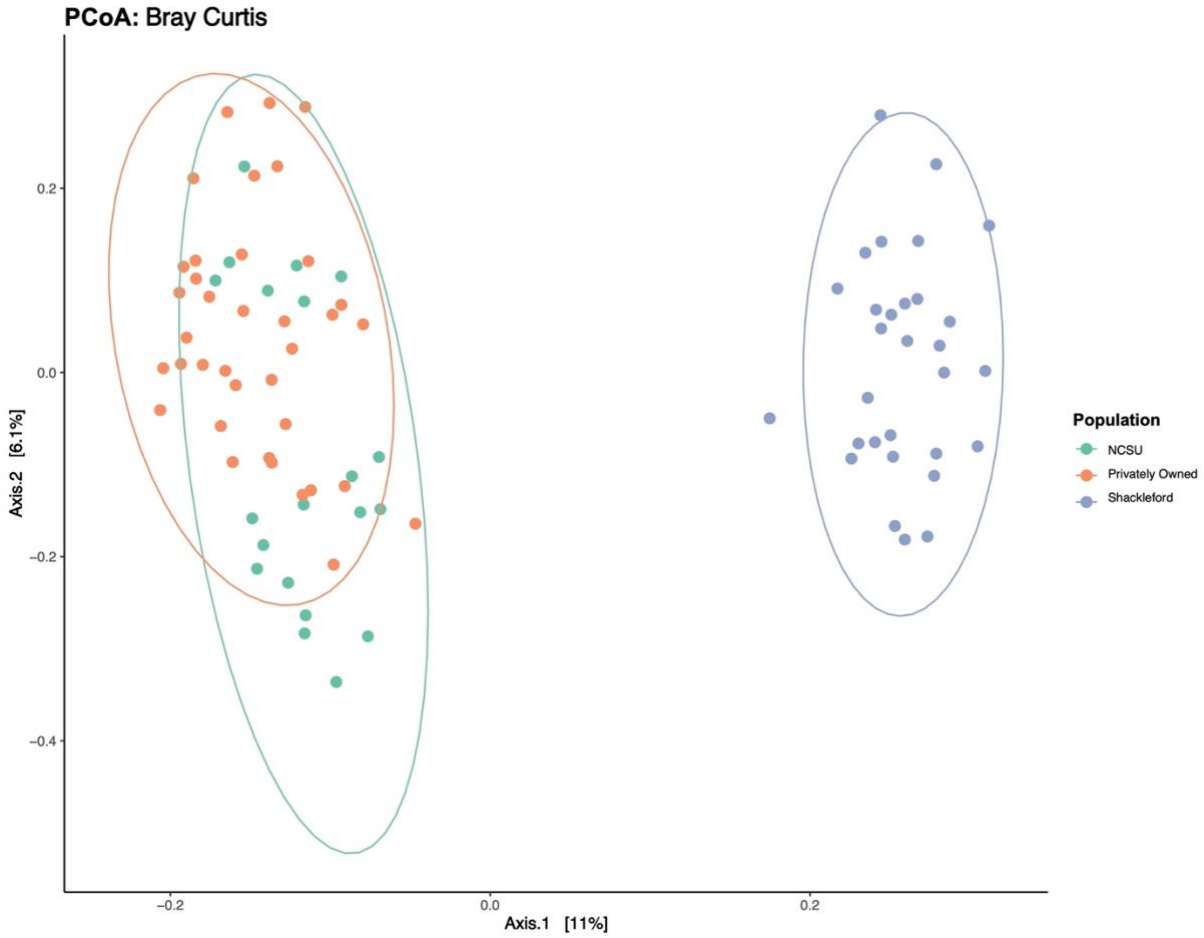

**Figure 2.6.** Principal coordinate analysis (PCoA) plot using the beta diversity metric Weighted UniFrac among the three populations (Shackleford, NCSU, privately owned). The percentage values within the brackets on Axis 1 and 2 refer to the percentage of explained variation

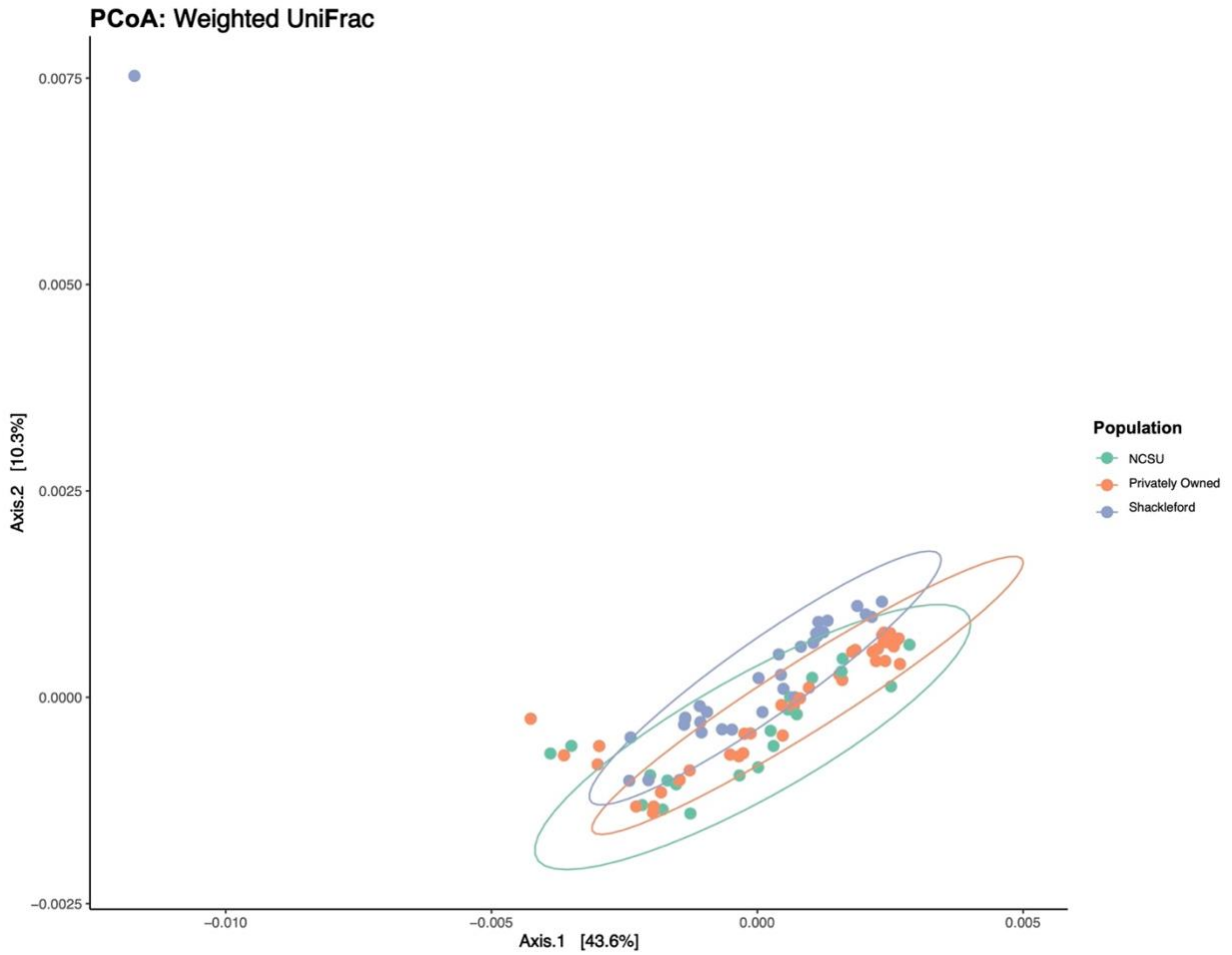

**Table 2.1.** Effect of equine population (Shackleford, NCSU, Privately Owned) and treatment (alfalfa, alfalfa plus starch) on methane (measured in nmol/ml) and pH values in *in-vitro* cultures of equine fecal inocula incubated at 39°C for 0, 2, 4 and 24 hours

| | | **Population** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Shackleford** | | **NCSU** | | **Privately Owned** | | | | | |
| | | **Treatments[1]** | | | | | | | **Fixed Effects, *P-value*[3]** | | |
| **Variables** | **Incubation (hours)** | **A** | **AS** | **A** | **AS** | **A** | **AS** | **SE[2]** | **Population** | **A vs. AS** | **Population* Treatment** |
| **Methane (nmol/ml)** | **0** | 7.8 | 7.9 | 8.0 | 9.8 | 11.3 | 12.7 | 0.91 | 0.002 | NS | NS |
| | **2** | 12.3 | 11.3 | 11.6 | 10.8 | 11.9 | 11.1 | 1.23 | NS | NS | NS |
| | **4** | 11.1 | 12.5 | 9.8 | 12.1 | 10.7 | 9.4 | 0.97 | NS | NS | NS |
| | **24** | 8.7 | 10.0 | 7.5 | 10.1 | 8.7 | 12.5 | 0.94 | NS | 0.006 | NS |
| **pH** | **0** | 6.3 | 6.4 | 6.3 | 6.3 | 6.4 | 6.3 | 0.002 | 0.003 | NS | 0.03 |
| | **2** | 6.7 | 6.6 | 6.3 | 6.2 | 6.2 | 6.5 | 0.016 | 0.008 | NS | NS |
| | **4** | 6.6 | 6.3 | 6.2 | 6.1 | 6.2 | 6.3 | 0.013 | 0.01 | NS | NS |
| | **24** | 6.0 | 5.6 | 5.6 | 5.3 | 6.2 | 5.8 | 0.024 | 0.004 | 0.005 | NS |

[1]Treatments consisted of: A (1 g alfalfa) and AS (0.8 g of alfalfa plus 0.2 g of starch)
[2]Standard error
[3]Statistical significance set at $P \leq 0.05$

**Table 2.2.** Effect of equine population (Shackleford, NCSU, Privately Owned) and treatment (alfalfa, alfalfa plus starch) on volatile fatty acids (acetate, propionate, butyrate and isoacids, expressed as molar %) and total VFAs (expressed as mM) in *in-vitro* cultures of equine fecal inocula incubated at 39°C for 0, 2, 4 and 24 hours

| | | **Population** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Shackleford** | | **NCSU** | | **Privately Owned** | | | | | |
| | | **Treatments[1]** | | | | | | | **Fixed Effects, *P-value*[3]** | | |
| **Variables** | **Incubation (hours)** | **A** | **AS** | **A** | **AS** | **A** | **AS** | **SE[2]** | **Population** | **A vs. AS** | **Population* Treatment** |
| **Acetate (molar%)** | **0** | 50.3 | 49.2 | 49.2 | 49.6 | 51.8 | 49.9 | 0.59 | NS | NS | NS |
| | **2** | 51.2 | 48.7 | 53.7 | 53.4 | 56.3 | 57.9 | 0.79 | <0.0001 | NS | NS |
| | **4** | 67.1 | 64.8 | 70.9 | 69.5 | 70.1 | 69.8 | 0.88 | 0.0005 | NS | NS |
| | **24** | 72.6 | 73.7 | 74.9 | 66.5 | 75.4 | 76.5 | 0.92 | 0.0004 | 0.018 | 0.0003 |
| **Propionate (molar%)** | **0** | 17.4 | 17.2 | 17.4 | 17.4 | 16.5 | 17.2 | 0.37 | NS | NS | NS |
| | **2** | 16.5 | 17.4 | 15.9 | 16.1 | 15.1 | 14.6 | 0.24 | <0.0001 | NS | 0.04 |
| | **4** | 9.6 | 10.9 | 9.8 | 10.2 | 10 | 10.2 | 0.29 | NS | 0.019 | NS |
| | **24** | 4.8 | 5 | 4.8 | 5.3 | 5.4 | 5 | 0.15 | NS | NS | NS |
| **Butyrate (molar%)** | **0** | 12.2 | 12.9 | 12.7 | 12.6 | 12.2 | 12.7 | 0.016 | NS | 0.03 | NS |
| | **2** | 12.3 | 12.9 | 11.5 | 11.6 | 10.9 | 10.5 | 0.018 | <0.0001 | NS | NS |
| | **4** | 11.6 | 11.7 | 7.9 | 8.4 | 7.9 | 7.9 | 0.031 | <0.0001 | NS | NS |
| | **24** | 16.4 | 15.2 | 14.5 | 21.9 | 12.8 | 12.7 | 0.064 | 0.0008 | 0.05 | 0.005 |
| **Isoacids (molar%)[4]** | **0** | 13.8 | 14.1 | 14.2 | 13.9 | 13.3 | 13.7 | 0.172 | 0.0086 | NS | NS |
| | **2** | 13.7 | 14.4 | 13 | 13 | 12 | 11.6 | 0.266 | <0.0001 | NS | NS |
| | **4** | 8 | 8.7 | 7.8 | 8.2 | 8.2 | 8.3 | 0.211 | NS | 0.04 | NS |
| | **24** | 4.6 | 4.3 | 4.3 | 4.5 | 4.9 | 4.2 | 0.299 | NS | NS | NS |
| **Total VFAs (mM)[5]** | **0** | 15.6 | 14.4 | 14.5 | 14.8 | 15.6 | 14.5 | 0.445 | NS | NS | NS |
| | **2** | 14.9 | 13.8 | 15.8 | 16 | 16.6 | 17.8 | 0.269 | <0.0001 | NS | 0.004 |
| | **4** | 25.4 | 26.1 | 28.7 | 26.4 | 26.6 | 26.8 | 0.89 | NS | NS | NS |
| | **24** | 60.2 | 52.4 | 63.7 | 52.1 | 59.8 | 60.6 | 2.601 | NS | 0.01 | NS |

[1]Treatments consisted of: A (1 g alfalfa) and AS (0.8 g of alfalfa plus 0.2 g of starch)
[2]Standard error
[3]Statistical significance set at $P \leq 0.05$
[4]Isoacids consisted of: isovalerate and isobutyrate
[5]Total VFAs (expressed as mM) consisted of: acetate, propionate, isobutyrate, butyrate, isovalerate and valerate

**Table 2.3.** Effect of equine population (Shackleford, NCSU, Privately Owned), treatment (alfalfa, alfalfa plus starch) and time (0, 2, 4, 24 hours) on methane (expressed as nmol/ml), pH, volatile fatty acids (acetate, propionate, butyrate and isoacids, expressed as molar %) and total VFAs (expressed in mM) in *in-vitro* cultures of equine fecal inocula incubated at 39°C

| | **Fixed Effects, *P-value*[4]** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Variables** | **Population[1]** | **Treatment[2]** | **Time[3]** | **Population* Treatment** | **Population* Time** | **Treatment* Time** | **Population* Treatment*Time** |
| **Methane (nmol/ml)** | NS | 0.035 | 0.003 | NS | 0.01 | 0.05 | NS |
| **pH** | <0.0001 | 0.005 | <0.0001 | NS | 0.0003 | 0.0006 | NS |
| **Acetate (molar%)** | <0.0001 | 0.0009 | <0.0001 | 0.01 | <0.0001 | NS | <0.0001 |
| **Propionate (molar%)** | 0.0003 | 0.022 | <0.0001 | NS | <0.0001 | NS | NS |
| **Butyrate (molar%)** | <0.0001 | 0.008 | <0.0001 | 0.005 | <0.0001 | NS | <0.0001 |
| **Isoacids (molar%)[5]** | <0.0001 | NS | <0.0001 | NS | <0.0001 | NS | NS |
| **Total VFAs (mM)[6]** | 0.028 | 0.003 | <0.0001 | 0.036 | NS | 0.0008 | 0.026 |

[1]Populations consisted of: Shackleford, NCSU and privately owned
[2]Treatments consisted of: A (1 g alfalfa) and AS (0.8 g of alfalfa plus 0.2 g of starch)
[3]Time consisted of incubation periods of: 0, 2, 4 and 24 hours
[4]Statistical significance set at $P \leq 0.05$
[5]Isoacids consisted of: isovalerate and isobutyrate

[6]Total VFAs (expressed as mM) consisted of: acetate, propionate, isobutyrate, butyrate, isovalerate and valerate

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# CHAPTER 3: STUDY 2

## Glucose and iron metabolism parameters in pasture horses

### 3.1. Abstract

Iron homeostasis is tightly regulated within the body. In equines, iron is primarily provided in a non-heme form through forages and grains. According to the National Research Council, iron recommendations are currently set at 400 mg daily for a 500 kg horse. This research aimed to assess the relationship between dietary iron and iron and glucose metabolism parameters in pasture-kept horses. Mixed-breed horses (n=21) kept on cool-season pasture for the previous 4 months were utilized. Body condition scores (BCS) (5.6±0.9) and body weight (496.2±56.41 kg) were recorded, jugular venous samples were taken and glucose (mg/dl) was measured using a hand-held glucometer. Samples were centrifuged and then serum was aliquoted and frozen at -20°C until analysis. Pasture samples were taken and analyzed for complete nutrient analysis in order to estimate daily iron intake (mg, DM basis). Serum samples were analyzed for insulin (using a commercial radioimmunoassay kit) and hepcidin (using an ELISA kit) while remaining samples were sent to various laboratories for analysis of ferritin, serum iron and total iron binding capacity (TIBC). Statistical analysis was performed in GraphPad Prism (GraphPad Software Version 10.2.0, Boston, MA). Correlation analysis using Pearson's correlation and descriptive statistics was performed. For horses with insulin values > 20 µU/ml, a separate Pearson's correlation was performed as well as simple linear regression to assess the relationship between insulin and ferritin concentrations. Significance was set at $P \leq 0.05$. Insulin and ferritin were not significantly correlated when considering all study horses (Pearson $r = 0.25$; $P = 0.2$). Forty-five percent of the horses were considered hyperinsulinemic and there was a significant correlation between insulin and ferritin concentrations (Pearson $r = 0.8$; $P = 0.01$).

Interestingly, the majority of horses had serum ferritin and iron concentrations above the published reference ranges, with a mean value of 409.2 ± 164.5 ng/ml and 174.8 ± 41.89 ug/dl, respectively. The results of this study warrant further investigation between serum insulin and ferritin concentrations, along with the impact of dietary iron.

### 3.2. Introduction

Iron (Fe) is an important micromineral and essential element for all living organisms (Andrews & Schmidt, 2007; Dev & Babitt, 2017). In the body, iron serves several functions such as oxygen transport and is a component of hemoglobin and myoglobin, with hemoglobin in red blood cells being the largest component of the body's iron at approximately 65% in horses, humans and other species (Dev & Babitt, 2017; Ems et al., 2017; Muñoz et al., 2009; Schryver, 1990). Iron homeostasis is tightly regulated within the body in order to maintain iron levels and prevent iron overload. Little iron from the diet is actually absorbed, anywhere from 5-35%, depending upon the type of iron as well as specific circumstances, for example in instances of iron deficiency (McDowell, 1992). Additionally, existing iron stores within the body control absorption and this is important as iron has no active excretion mechanism; thus, the reason iron is so tightly regulated in order to maintain homeostasis (Dev & Babitt, 2017).

When iron is stored, which accounts for 25-26% of total body iron, it is primarily stored as ferritin (Andrews & Schmidt, 2007). Ferritin is a storage protein that is synthesized and stores iron within hepatocytes in the liver. If iron is needed, ferritin can easily be mobilized to export iron out of the cell via the basolateral exporter ferroportin in order to be circulated (via transferrin) through the body as needed (Dev & Babitt, 2017; Donovan et al., 2000). Serum ferritin has been thought to be the best way to measure levels of iron within the body in both

humans (Abbaspour et al., 2014) and horses. In horses, Smith et al. (1984) found serum ferritin levels to range from 70-250 ng/ml in mature adult horses (Smith et al., 1984).

Hepcidin is the master regulator of systemic iron homeostasis, a peptide hormone primarily produced mainly in the liver by hepatocytes, although it has been shown to be expressed in other tissues like adipose (Bekri et al., 2006) and the heart (Camaschella et al., 2020; Yiannikourides & Latunde-Dada, 2019). Hepcidin primarily controls iron within the circulation based on the overall body status of iron. If iron is high, hepcidin expression will be increased, ultimately resulting in less iron being circulated, which is done by hepcidin binding to the exporter ferroportin and degrading it. If iron is low, hepcidin expression will be reduced, allowing ferroportin to function properly and letting iron into the bloodstream (Andrews & Schmidt, 2007; Dev & Babitt, 2017; Nicolas et al., 2002). Thus, hepcidin is an important player in maintaining iron homeostasis in times of high iron (e.g.., iron overload) and/or low iron (e.g., iron deficiency).

In horses, dietary iron is generally provided as non-heme iron. This dietary form of iron is most common in plants (e.g., forages and grains), although it is less bioavailable than heme iron, which is found in meat products (Ems et al., 2017; Hurrell & Egli, 2010). Forages will generally contain 100-250 mg per kg DM of iron (NRC, 2007), however iron levels within hay have been reported to be much higher, with Richards and Nielsen (2018) finding grass hay contains anywhere from 31-3760 mg/kg of iron (Richards & Nielsen, 2018). Potential reasons for higher levels of iron in forages, especially hay, is likely due to soil and machinery contamination (Geor et al., 2013). Although endogenous iron losses have not been reported in horses, the National Research Council currently has daily iron requirements set at 400 mg for a 500 kg horse at maintenance (NRC, 2007). It is important to note this recommendation hasn't

been changed since 1989 due to lack of research within this area (NRC, 1989, 2007). Maximum tolerable concentrations are currently set at 500 mg/kg of ration based on data from other species (NRC, 2006), although a survey-based study has found that racing Thoroughbreds consume 3900 mg of iron daily just from hay and grain (McLean et al., 2022), indicating that most horses are likely consuming well over the daily iron recommendations with no adverse effects reported.

Due to the limited research done assessing iron metabolism within horses, information on the potential relationship between dietary iron and iron and glucose metabolism parameters is lacking. Therefore, this study aimed to analyze dietary iron intake, glucose metabolism parameters (e.g., basal insulin, glucose) and iron metabolism parameters (e.g., ferritin, serum iron, total iron binding capacity, hepcidin) to assess if there are relationships among these in a population of horses kept on pasture.

## 3.3. Materials and Methods

The North Carolina State University Institutional Animal Care and Use committee approved this study (Protocol #23-173-01).

### *3.3.1. Animals*

All horses used for the study were housed at the North Carolina State University Reedy Creek Farm and kept on cool-season mixed pasture for the previous 4 months of the study. If horses were supplemented with additional hay and/or concentrates, this was noted. Additionally, all horses had access to free choice white salt and automated waterers. Horses included in the study (n=21) were mixed-breed and included geldings, stallions and mares, with body weight ranging from 385 - 625 kg.

*3.3.2. Procedure*

All sampling for the study was completed in the morning to ensure glucose and insulin levels were not affected by potential starches and/or sugars within the diet. Horses had access to pasture and/or hay overnight.

Two researchers evaluated each horse and assigned a Body Condition Score (BCS) based on the 1-9 scoring system developed by Henneke (Henneke et al., 1983) and these scores were averaged while body weight (BW, kg) assessed via weight tape were recorded from health records. Jugular venous samples were then taken in vacutainer serum tubes containing no anticoagulant (BD Company, Franklin Lakes, NJ), collecting approximately 30 ml. Immediately after blood collection, glucose was measured from fresh whole blood samples using a hand-held glucometer (One Touch Ultra 2, LifeScan Inc., Malvern, PA) and glucose test strips (OneTouch Ultra Test Strips, LifeScan Inc., Malvern, PA). The use of a hand-held glucometer for glucose assessment in horses has been previously validated (Hackett & McCue, 2010). Blood samples collected in serum vacutainer tubes were allowed to clot and then centrifuged at 1200 x *g* at 4°C for approximately 15 minutes. Upon completion of centrifugation, serum was aliquoted into microcentrifuge tubes (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA) and frozen at -20°C until analysis.

Based on the BCS, glucose levels and availability of the initial 21 horses sampled, 12 mixed-breed geldings were selected to continue onto another study, detailed in subsequent chapters.

Dietary information was collected for each horse in order to determine total iron intake. Representative pasture samples were taken of each horse(s)' pasture and sent to the North Carolina Department of Agriculture & Consumer Services (NCDA&CS, Raleigh, NC) Forage

Testing for complete analysis, which included moisture, protein, fiber, minerals (including iron) and other analytes. Representative hay samples were also taken and analyzed by NCDA&CS. If horses were consuming any concentrates (n=10), the daily amount was recorded as well as the iron content (verified by the feed tag).

*3.3.3. Sample Analysis*

Serum samples were analyzed for ferritin, serum iron, total iron binding capacity (TIBC), insulin and hepcidin.

*3.3.3 a. Serum Ferritin, Iron, Total Iron Binding Capacity (TIBC)*

Analysis for serum ferritin was completed at the Kansas State Veterinary Medical Diagnostic Laboratory (KVMDL, Manhattan, KS). Serum ferritin was measured in ng/ml, with KVMDL noting their adult reference range for horses is 43-261 ng/ml based upon testing data and previous research done by Smith et al. (1984), which noted in their original study a reference range of 70-250 ng/ml (Smith et al., 1984).

Serum iron and TIBC analysis was completed at the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) and measured by automated analyzer. Reference ranges provided by TVMDL are 73-140 ug/dl and 306-518 ug/dl for serum iron and TIBC, respectively.

*3.3.3 b. Insulin*

Insulin concentrations were measured in serum samples. A commercial Human-Insulin Specific Radioimmunoassay kit was used (Kit #HI-14K, EMD Millipore Corporation, Billerica, MA). The sensitivity of the assay is 2.72 μU/ml per the manufacturer. This kit has been previously validated for use in horses (Spears et al., 2020) but was also validated by the Pratt-Phillips laboratory at North Carolina State University. Validation was done by dilutional

parallelism between kit standards and linearity in equine serum previously assessed for insulin. Samples were run in duplicate and samples were repeated on subsequent runs if CV > 15%.

*3.3.3 c. Hepcidin*

Hepcidin concentrations were measured in serum samples. A Horse Hepcidin-Compete Enzyme Linked Immunosorbent Assay (ELISA) kit not yet available for commercial use was used (Intrinsic LifeSciences LLC, La Jolla, CA). The kit was validated by the Pratt-Phillips laboratory at North Carolina State University by dilutional parallelism between kit standards and recovery. All samples were diluted 10% based on kit instructions and run in duplicate. Samples were repeated on subsequent runs if CV >15%.

*3.3.4. Statistical Analysis*

Statistical analysis was performed in GraphPad Prism (GraphPad Software, Version 10.2.0, Boston, MA). Descriptive statistics (mean, standard deviation, ranges) were calculated, with means presented as mean ± standard deviation (SD). Insulin: Glucose (I/G) and Glucose: Insulin (G/I) ratios were also calculated. Pearson's correlation analysis was performed on the variables BCS, BW, insulin, glucose, ferritin, serum iron, TIBC, ferritin and iron intake, with the association between variables determined based on the correlation coefficient and P-value, with a significance level of $P \leq 0.05$. A trend was noted if $0.05<P\leq 0.1$ For horses with basal insulin concentrations > 20 μU/ml, an additional Pearson's correlation was performed to assess the association between variables insulin and ferritin. Simple linear regression was also performed between these two variables.

**3.4. Results**

Means, standard deviations (SD) and ranges of body condition score, body weight and concentrations of variables are presented in Table 3.1. The majority of horses (66%, 14/21) had

body condition scores in the ideal range (BCS 4-6) while the remaining horses (33%, 7/21) had scores that would be considered fleshy or obese (BCS > 6). There was a large range for estimated iron intake, in which horses were consuming anywhere from 1596 – 31675 mg daily on a DM basis (Table 3.1).

A correlation matrix of the variables assessed, excluding the I/G and G/I ratio, is shown in Figure 3.1. There was a moderate, positive relationship between serum iron and TIBC (Pearson r = 0.44; P=0.05), iron intake and TIBC (Pearson r = 0.46; P = 0.04) and iron intake and hepcidin (Pearson r = 0.57; P = 0.01). There was a moderate, negative relationship between BCS and hepcidin (Pearson r = -0.57; P = 0.01) as well as between BCS and iron intake (Pearson r = -0.54; P = 0.01). Trends were noted for relationships between ferritin and hepcidin (Pearson r = 0.35; P = 0.1), ferritin and TIBC (Pearson r = 0.37; P = 0.1), ferritin and serum iron (Pearson r = 0.32; P = 0.1) and serum iron and hepcidin (Pearson r = -0.42; P = 0.08). Finally, the correlation between insulin and ferritin across all study horses was not significant (Pearson r = 0.25; P = 0.2) (Figure 3.1).

Forty-five percent (45%, 9/20) of the study horses had a basal insulin concentration of > 20 µU/ml, which has been used previously for a horse to be considered hyperinsulinemic (Durham et al., 2019). When assessing the correlation between insulin of horses considered hyperinsulinemic (n=9) and ferritin concentrations, Pearson's correlation found a strong, positive relationship between these two variables (Pearson r = 0.8; P = 0.01). Additionally, simple linear regression also found the relationship between these two variables to be significant (P = 0.01) (Figure 3.2).

The reference range for serum ferritin concentrations provided by KSVDL was 43-261 ng/ml, which is based on studies done by Smith et al. (1984) (Smith et al., 1984). The majority of

horses (85%; 18/21) included in the study had ferritin concentrations exceeding this reference range. Finally, reference ranges for serum iron and TIBC were provided by TVMDL and were 73-140 ug/dl and 306-518 ug/dl for serum iron and TIBC, respectively. Most of the horses (80%, 16/20) had serum iron levels over the provided reference range. For TIBC, all the horses had TIBC concentrations within the reference range except 1.

**3.5. Discussion**

Results showed that the majority of horses had elevated serum ferritin and iron concentrations compared to reference ranges. Additionally, 9 horses had basal insulin concentrations indicative of hyperinsulinemia based on a cut-off value of >20 μU/ml (Durham et al., 2019) out of the 21 assessed. Finally, there were some moderate but significant correlations between serum iron and TIBC as well as between iron intake and some iron parameters (e.g., TIBC, hepcidin), indicating there are potential correlations between dietary iron intake and important iron metabolism parameters such as hepcidin.

There have been limited studies assessing iron metabolism in relation to dietary iron in horses but studies that have been done have yielded interesting results (Lawrence et al., 1987; Pearson & Andreasen, 2001). In a study by Pearson and Andreasen that supplied excess dietary iron for 8 weeks in ponies, serum iron and ferritin concentrations increased but no adverse clinical effects were found. However, Pearson and Andreasen utilized an inorganic form of iron, ferrous sulfate, to supplement the diet so ponies received 2500 μg/kg of elemental iron (Pearson & Andreasen, 2001), while the present study primarily supplied iron in natural feeds. Additionally, it is important to note the present study did not manipulate the diet at all, rather samples were taken at specific time points to assess glucose and iron metabolism parameters as a baseline for subsequent studies. Although Pearson and Andreasen saw elevated concentrations of

serum iron and ferritin when supplementing iron via ferrous sulfate, a study by Lawrence et al. (1987) failed to see an increase in serum iron when supplementing ponies with high levels of iron (Lawrence et al., 1987).

Information on hepcidin, specifically serum hepcidin concentrations, is limited in horses. In humans, serum hepcidin levels in healthy individuals has been found to range from 76 – 108 ng/ml (Girelli et al., 2009). Hepcidin mRNA has been identified in liver tissue and expression of it has been shown to be upregulated when inflammation is induced in horses (Oliveira Filho et al., 2010; Oliveira-Filho et al., 2012; Oliveira-Filho et al., 2014). This is line with other species, like humans, in which hepcidin expression has been shown to be influenced by inflammation as hepcidin is an acute phase protein (Dev & Babitt, 2017; Grimes et al., 2012). Reference ranges for serum hepcidin levels in horses have not been established, but a few studies have evaluated serum hepcidin (Satué, Fazio, Cravana, et al., 2023; Satué, Fazio, La Fauci, et al., 2023; Wijekoon et al., 2022). A study of Mongolian horses found a serum hepcidin concentration of $110 \pm 40$ ng/ml, however injection of pentosan polysulfate, an injectable used to treat osteoarthritis, reduced hepcidin concentrations (Wijekoon et al., 2022). In pregnant mares, serum hepcidin concentrations decreased throughout pregnancy while serum ferritin and serum iron increased (Satué, Fazio, Cravana, et al., 2023). In the present study, hepcidin concentrations ranged from 11.82 – 91.48 ng/ml. These concentrations are slightly lower but similar to previous studies that assessed equine serum hepcidin, however these differences could be due to a different ELISA kit used (Satué, Fazio, Cravana, et al., 2023; Wijekoon et al., 2022).

Both serum ferritin and iron were elevated in the present study based on the provided laboratory reference ranges. In a study of exercising horses and its subsequent effects on iron metabolism done by Inoue et al. (2005), serum iron generally decreased across the weeks of

exercise, with values of 1.74 ± 0.11 mg/l (equivalent to 174 ± 11 ug/dl) before exercise and values of 1.19 ± 0.23 mg/l (equivalent to 119 ± 23 ug/dl) after exercise (Inoue et al., 2005). Serum iron during the pre-exercise period was similar to the present study, in which serum iron values were 174.8 ± 41.89 ug/dl. However, it is important to note that the present study did not exercise the horses and that hemolysis is likely playing a role in altered iron status during exercise, thus why Inoue et al. (2005) likely saw a reduction in serum iron (Inoue et al., 2005).

Ferritin concentrations and hyperinsulinemia have been found to be correlated in some studies (Kellon & Gustafson, 2019; Nielsen et al., 2012). When assessing all horses within the study, there was no significant correlation between insulin and ferritin values. However, when looking only at horses with elevated basal insulin concentrations of >20 µU/ml (n=9), the correlation between ferritin and insulin was significant (Figure 3.2). Nielsen et al. (2012) utilized horses as a model animal for black rhinoceros as captive rhinos are susceptible to iron overload and possess similar digestive systems. There was a positive correlation between elevated ferritin and insulin responses after dextrose and pelleted corn administration, indicating a potential, positive connection between body iron stores and insulin resistance (Nielsen et al., 2012). Additionally, Kellon and Gustafson (2019) produced a similar result, who found that 100% of hyperinsulinemic horses had ferritin values over the published reference range of 43-261 ng/ml (Kellon & Gustafson, 2019; Smith et al., 1984). However, this published reference range for ferritin is from research done 40 years ago, thus there may be a need for updated ranges as equine diets have changed since then. Ultimately, it does seem plausible that elevated body iron stores and insulin resistance could be related, although this relationship does need to be explored further.

Despite the relationships seen in the present study, there were some limitations. Iron intake was estimated based on the assumption that horses were consuming 2% of their body weight in pasture and forages daily. However, it is estimated that grazing horses generally consume 1.5 - 3.1% of their body weight in voluntary dry matter intake (NRC, 2007) so some horses could have been consuming more or less iron daily. Additionally, estimated dietary iron intake varied widely (1596 – 31675 mg, DM basis), however iron levels have been shown to vary widely in grasses and forages due to soil contamination (Geor et al., 2013). Another limitation was that jugular venous samples were only taken at one time point, which could lead to misleading values of iron parameters, for example ferritin. Ferritin is constantly turned over as it has a life span of just a few days, thus the measure of ferritin, a reflection of body iron stores, could be different based on where ferritin is at in being turned over (Kamerling & Tobin). However, it is generally regarded that serum ferritin is the best way to determine body iron stores in horses and other species (Abbaspour et al., 2014; Smith et al., 1984).

### 3.6. Conclusion

Elevated serum ferritin and iron concentrations were seen amongst the study population. Additionally, 45% of horses were considered hyperinsulinemic with basal insulin values of >20 µU/ml and there was a significant correlation between insulin and ferritin concentrations. Of general interest is the overall elevated ferritin concentrations in this specific equine population compared to published reference ranges. There were also moderate relationships seen between iron parameters (e.g., hepcidin) and iron intake as well as serum iron and TIBC. Serum hepcidin concentrations were also determined, although there is limited work done in this area within horses. Overall, it does seem there are some relationships between iron and glucose metabolism parameters in horses kept on pasture.

## Tables and Figures

**Table 3.1**. Means, standard deviations and ranges of variables measured in mixed-breed horses (n=21) kept on cool-season mixed pasture for the previous four months

| Variable | Mean ± Standard Deviation | Range |
|---|---|---|
| Body Condition Score | 5.6 ± 0.90 | 4.2 - 7 |
| Bodyweight (kg) | 496.2 ± 56.41 | 385 - 625 |
| Insulin (µU/ml) | 19.5 ± 10.00 | 6.98 – 40.62 |
| Glucose (mg/dl) | 85.8 ± 9.80 | 69 - 110 |
| I/G Ratio | 0.2 ± 0.13 | 0.082 – 0.549 |
| G/I Ratio | 5.8 ± 3.09 | 1.822 – 12.22 |
| Ferritin (ng/ml) | 409.2 ± 164.50 | 183 - 747 |
| Serum Iron (ug/dl) | 174.8 ± 41.89 | 80.1 - 287 |
| TIBC (ug/dl) | 423.6 ± 53.33 | 323.3 – 555.3 |
| Hepcidin (ng/ml) | 47.8 ± 18.85 | 11.82 – 91.48 |
| Estimated Iron Intake (mg) | 11334.0 ± 10090.00 | 1596 - 31675 |

**Figure 3.1.** Correlation matrix showing Pearson correlation coefficients (r) between different variables assessed in mixed-breed horses (n=21) kept on cool-season mixed pasture for the previous four months. Cells are colored according to whether the correlations are positive (blue) or negative (red) and shaded according to the strength of the relationship based on the Pearson correlation coefficient. Statistical significance was set at $P \leq 0.05$ while a trend was noted if $0.05 < P \leq 0.1$

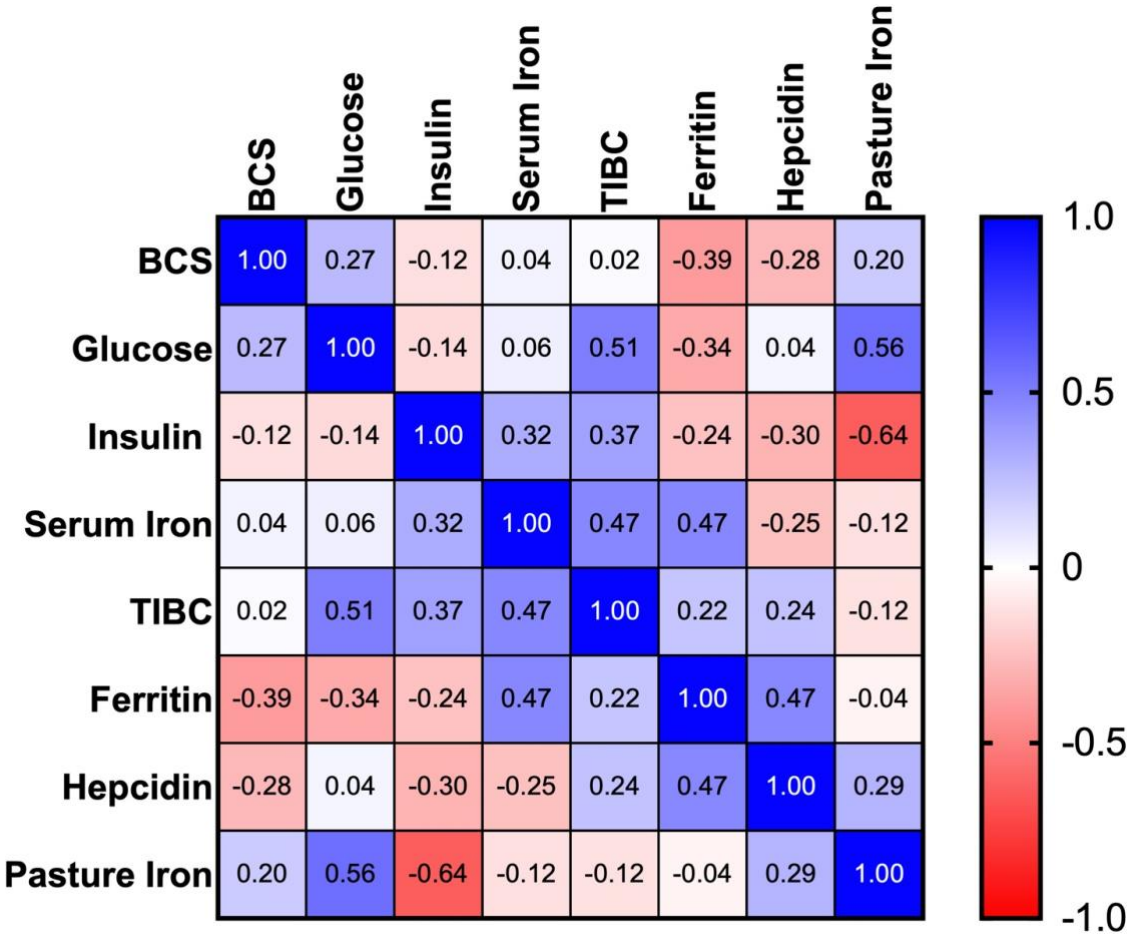
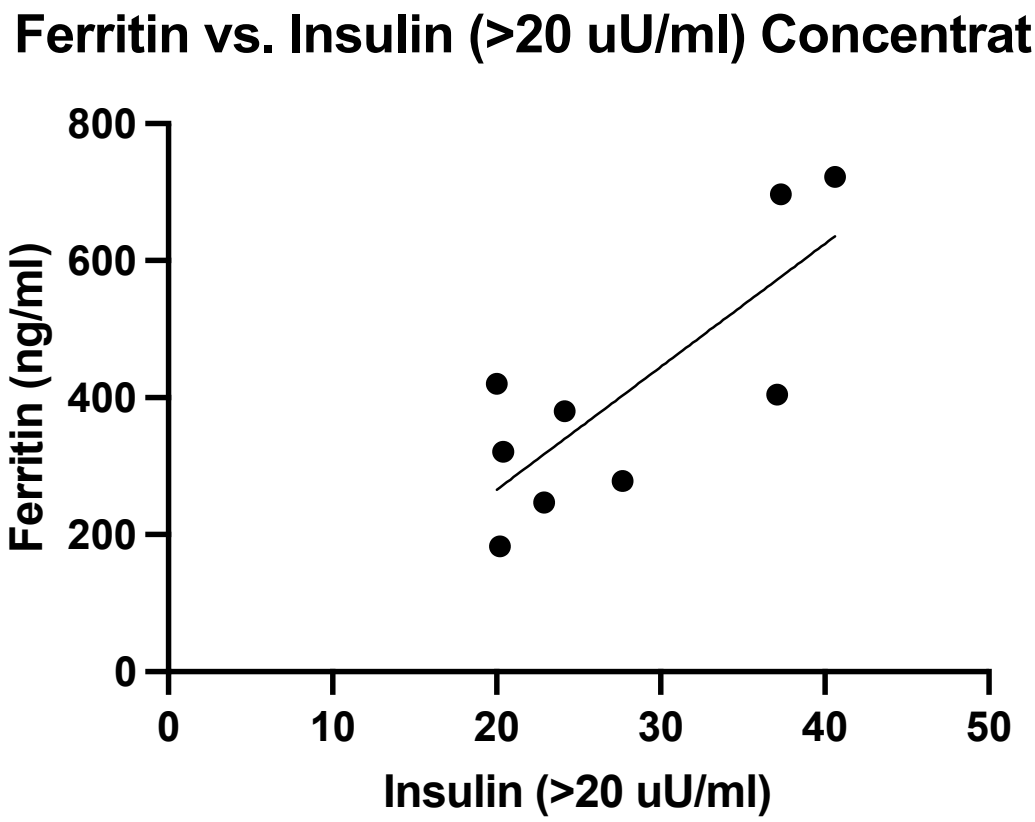


**Figure 3.2.** Simple linear regression plot of mixed-breed horses (n=9) kept on cool-season mixed pasture for the previous four months considered hyperinsulinemic (basal insulin concentrations >20 uU/ml) (denoted on the x-axis) and ferritin concentrations (denoted on the y-axis). Statistical significance was set at $P \leq 0.05$ while a trend was noted if $0.05 < P \leq 0.1$



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# CHAPTER 4: STUDY 3

## The effects of iron supplementation on glucose and iron metabolism parameters

### 4.1. Abstract

Iron (Fe) is an essential element for all living organisms and is tightly regulated within the body, with ferritin being an indicator of overall body iron status. Additionally, previous studies have pointed to a possible relationship between elevated body iron stores and insulin resistance. Thus, the objective of the study was to assess the effects of iron supplementation on iron and glucose metabolism parameters as well as digestibility. Mixed-breed geldings (n=12; BW 546.43±74.43 kg) were utilized for the study, being housed individually and consuming 2% of their body weight in mixed-grass hay and an iron-free vitamin mineral supplement. Horses consumed 481.64±76.95 mg Fe on a DM basis daily for the first 28 days (Hay Phase), which is similar to the NRC's daily iron recommendations of 400 mg daily for a 500 kg horse. At the end of the Hay Phase, an oral sugar test (OST) was conducted to assess insulin and glucose responses after being given a 0.45 ml/kg BW dose of Karo Light Syrup. Horses also underwent a 24-hour total fecal collection. In the next 28 days of the study (Supplement Phase), horses were either assigned to continue on the same hay diet (CTRL; n=4) or receive a daily iron supplement (IRON; n=8) in the form of ferrous sulfate, with IRON horses consuming 3942.99±424.35 mg Fe daily on a DM basis. Another OST and 24-hour total fecal collection was completed. Serum samples were analyzed for various iron metabolism indicators as well as insulin, complete blood counts were measured and fecal samples were analyzed for fecal iron. Statistical analysis was performed in GraphPad Prism Version 10.2.0 (GraphPad Software, Boston, MA) utilizing correlation analysis and analysis of variance for repeated measures. Serum ferritin was significantly higher in IRON horses across the 2 phases (P=0.01), in which values were

522.5±189.48 ng/ml at the end of the study. Although 60-min insulin values were not indicative of insulin dysregulation (>65 μU/ml) in IRON horses, insulin responses were significantly higher compared to CTRL horses. Additionally, an unpaired t test found 60-min insulin to ferritin values to be significant (P<0.0001) in IRON horses. Finally, fecal iron excretion was higher (P=0.002) in IRON horses between the Hay and Supplement Phase. Results potentiate that there is a possible relationship between insulin response and body iron status.

**4.2. Introduction**

Iron is an essential micromineral involved in several physiological functions throughout the body, one of them being oxygen transport (Grimes et al., 2012). Hemoglobin, a protein found in red blood cells, is where the majority of iron is distributed within the body at about 65% or 2 g of iron (Dev & Babitt, 2017). Iron can also exist as storage iron compounds such as ferritin and hemosiderin, constituting about 25-26% of body iron. However, the majority of iron will be stored as ferritin in the body (Andrews & Schmidt, 2007). Ferritin is an intracellular iron storage protein synthesized within the hepatocytes in the liver and it can hold up to 4500 ions of iron (Camaschella et al., 2020; Yiannikourides & Latunde-Dada, 2019). By storing iron within the body as ferritin, it allows for tighter control of iron delivery to the exporter ferroportin, where it can then be released into the bloodstream to be circulated and mobilized as needed (Andrews & Schmidt, 2007; Dev & Babitt, 2017; Donovan et al., 2000). Ferritin is thought to be the best indicator of body iron status by measuring serum ferritin levels (Abbaspour et al., 2014). Serum ferritin levels in horses at maintenance have been established, with Smith et al. (1984) reporting a range of 70 - 250 ng/ml (Smith et al., 1984).

Another important player within iron metabolism is hepcidin, which is synthesized in the liver. Hepcidin is a peptide hormone that is thought to be the master regulator of systemic iron

homeostasis. Hepcidin acts by controlling the amount of iron within the circulation through a negative feedback mechanism, with hepcidin expression depending upon circulating and tissue iron (Camaschella et al., 2020). Although limited information is available on hepcidin in horses, hepcidin has been sequenced in horses and its expression has been found to be upregulated in times of inflammation, similar to humans (Grimes et al., 2012; Oliveira Filho et al., 2010; Oliveira-Filho et al., 2012). While there has been no research to the author's knowledge in equines on the relationship among iron indicators (e.g., ferritin, hepcidin), obesity and metabolic issues, like insulin resistance, studies in humans have found that obesity is associated with increased serum ferritin and hepcidin expression (Jehn et al., 2004; Moore Heslin et al., 2021; Vuppalanchi et al., 2014). Iron has been shown to affect glucose and lipid metabolism in humans, in which iron is taken up by adipocytes due to insulin, resulting in increased body iron stores and ultimately an increased risk for insulin resistance (Dev & Babitt, 2017; Ford & Cogswell, 1999). Studies in horses have seen a correlation between elevated body iron status and insulin response by using an oral drench of dextrose, in which horses with higher insulin responses also had elevated ferritin levels (Nielsen et al., 2012). Thus, it seems plausible that body iron status and glucose metabolism are interrelated.

Per the National Research Council, iron requirements are currently set at 400 mg for a 500 kg horse at maintenance (NRC, 2007). However, maximum tolerable concentrations are based on data from other species and currently set at 500 mg/kg of ration (NRC, 2006). Horses are often consuming well over the daily iron recommendations, with a survey-based study finding that racing Thoroughbreds (n=1978) consumed 3900 mg of iron daily from hay and grain alone, with iron-specific supplements adding an additional 500 mg of iron, with these supplements most often being used for their perceived performance benefits (McLean et al.,

2022). In their survey-based study, McLean et al. (2022) also reported no insulin resistance being seen in the population of horses, indicating that dietary iron is likely not a cause of insulin resistance (McLean et al., 2022). However, this conclusion needs to be taken with caution as racing Thoroughbreds likely have a lower incidence of insulin resistance compared to other equine populations (Breuhaus, 2019; Pratt-Phillips et al., 2010).

Obesity and subsequent metabolic issues are concerns amongst horse owners as obesity is prevalent in equine populations, with Rendle et al. (2018) estimating that obesity rates may be as high as 70% (Rendle et al., 2018). Additionally, iron intake within the equine diet has become a "hot topic" amongst horse owners, in which 40 "hits" were found using the word "iron" in a popular Facebook group called Equine Nutrition Education (https://www.facebook.com/groups/equinenutritioneducation). Studies in humans have explored the relationship between obesity and iron, in which iron can be taken up by adipose tissue, resulting in elevated serum ferritin and an increased risk for insulin resistance (Dev & Babitt, 2017; Ford & Cogswell, 1999; Jehn et al., 2004; Moore Heslin et al., 2021). Thus, with increasing concerns about obesity as well as dietary iron often seen in equine diets, this relationship needs to be explored further within horses.

The present study aimed to assess the effects of iron supplementation on iron metabolism parameters such as serum iron and ferritin. Additionally, by utilizing an oral sugar test, a well-known field-based diagnostic tool that has been shown to effectively assess insulin dysregulation (Schuver et al., 2014), the present study also aimed to measure glucose and insulin responses in horses supplemented with iron to see if there are any differences between horses fed at maintenance levels of iron versus excess iron. Finally, a 24-hour fecal collection was utilized to assess the effects of iron supplementation on fecal iron excretion. It was hypothesized that iron

supplementation would result in increased iron metabolism parameters (e.g., ferritin, serum iron) and insulin responses.

## 4.3. Materials and Methods

The North Carolina State University Institutional Animal Care and Use committee approved this study (Protocol #23-173-01).

### *4.3.1. Animals*

All horses used for the study were housed at the North Carolina State University Reedy Creek Farm and kept on cool-season mixed pasture for the previous 4 months prior to the study. Additionally, all horses had access to free choice white salt and automated waterers. Horses used in the study were mixed-breed geldings (n=12) with a mean ± standard deviation (SD) body weight (BW) of 546.4±74.43 kg and body condition scores (BCS) ranging from 4.2-7 and a mean value of 5.7±0.9. The 1-9 scale for Body Condition Score (BCS) developed by Henneke was utilized for the study (Henneke et al., 1983). All horses were weighed using a livestock scale (Gallagher 210 Livestock Scale, Gallagher USA, Riverside, MO) at the beginning of the study. Horses were re-weighed approximately every 7 days during the initial study period and feed adjustments were made as necessary if there were body weight changes.

### *4.3.2. Procedure*

Horses were housed in individual, partially-covered, 3.7 x 12.2-meter dry lot pens with automatic waterers and had access to free-choice salt. Water samples were taken, sent to the North Carolina Department of Agriculture and Consumer Services (NCDA&CS, Raleigh, NC) Agronomic Division and was determined to contain 0.04 ppm of iron. Horses were fed an orchardgrass-timothy hay. A representative hay sample was also taken and sent to the NCDA&CS (Raleigh, NC) for complete nutrient analysis (Table 4.1) and contained 53.63 ppm

of iron on a dry matter (DM) basis. At the start of the study, horses were offered 2% of their body weight in hay, however refusals were recorded and hay offered was adjusted based on this, thus horses were offered anywhere from 1.5-2% of their body weight in hay throughout the study.

In order to ensure all nutrient requirements were met based on the National Research Council recommendations for mature horses at maintenance (NRC, 2007), an iron-free vitamin mineral supplement, SmartVite Thrive Pellets (SmartPak Equine, Plymouth, MA) was provided at 1 scoop daily, or approximately 40 g (Table 4.2). Additionally, powdered Vitamin E (200 IU), zinc (100 mg) and copper (1500 mg) were provided (Bulk Supplements, Henderson, NV).

The study was divided into two phases, called the Hay Phase and Supplement Phase. The first 28 days of the study (Hay Phase), was an acclimation period in which all horses were consuming the same diet. At the end of this phase, an oral sugar test (OST) was performed, which is detailed further below, as well as a hematology cell count test. Additionally, a 24-hour total fecal collection was performed, which is detailed further below.

For the latter 28 days of the study (Supplement Phase), horses were randomly assigned into 2 groups, CTRL (Control; n=4) or IRON (Supplement; n=8). Both CTRL and IRON horses consumed 1.5-2% of their body weight in orchardgrass-timothy hay daily. A representative hay sample was taken and sent to the North Carolina Department of Agriculture & Consumer Services (NCDA&CS, Raleigh, NC) for complete nutrient analysis (Table 4.3) and contained 74.11 ppm of iron on a dry matter (DM) basis. The reason the iron content of this hay was slightly higher was due to a different hay supply being utilized for the latter 28 days of the study. Horses in the CTRL group remained on the same hay and vitamin-mineral supplement while IRON horses received an iron supplement. The iron supplement in the form of ferrous sulfate

was administered orally daily. The ferrous sulfate was a powdered-form and classified as animal feed grade (Alpha Chemicals, Missouri, USA). As the ferrous sulfate was a powder, it was weighed out and mixed thoroughly with sugar-free applesauce before being administered to horses in an oral dosing syringe every morning. The iron dose used was 3000 mg/500 kg BW or 6 mg/kg BW. This dose was determined based on previous work done in which ponies administered 50 mg of iron per kg of BW in the form of ferrous sulfate for 8 weeks displayed no adverse clinical effects (Pearson & Andreasen, 2001). As ferrous sulfate only contains 20% elemental iron, this was factored in when calculating the daily dosage. At the end of the Supplement Phase, another OST, hematology cell count test and 24-hour total fecal collection were performed.

*4.3.2 a. Oral Sugar Test (OST)*

An oral sugar test (OST) was conducted two times throughout the study. Horses were fasted overnight and the test was performed beginning at 7:00 AM. Time 0 jugular venous samples were taken in vacutainer serum tubes containing no anticoagulant (BD Company, Franklin Lakes, NJ), collecting approximately 20 ml. Immediately after blood collection, glucose was measured from fresh whole blood samples using a hand-held glucometer (One Touch Ultra 2, LifeScan Inc., Malvern, PA) and glucose test strips (OneTouch Ultra Test Strips, LifeScan Inc., Malvern, PA). The use of a hand-held glucometer for glucose assessment in horses has been previously validated (Hackett & McCue, 2010).

A 0.45 ml/kg of BW dose of Karo Light Corn Syrup (ACH Food Companies, Inc., Oakbrook Terrace, IL) was selected based on recommendations by the Equine Endocrinology Group and previous research as this higher dose can improve test performance (Frank et al., 2022; Jocelyn et al., 2018). Individual horse doses were calculated based on body weight and

were administered orally via a dosing syringe, ensuring that minimal Karo syrup was spilled. After 60 minutes, subsequent jugular venous samples were collected in serum vacutainer tubes (BD Company, Franklin Lakes, NJ) and glucose was measured using a glucometer. Blood samples collected in serum vacutainer tubes were allowed to clot then centrifuged at 1200 x *g* at 4°C for approximately 15 minutes. Upon completion of centrifugation, serum was aliquoted into microcentrifuge tubes (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA) and frozen at -20°C until analysis.

*4.3.2 b. Fecal Collection*

A 24-hour total fecal collection was conducted at the end of the Hay and Supplement Phase. Prior to the collection, horses were fitted with a Bun-Bag Manure Catcher and Surcingle Attachment (Bun-Bag Horse Diapers, Idaho, USA) (Figure 4.1).

Fecal collections began at 12 PM and ended 24 hours later. Horses were provided their feed as normal and the manure catchers were emptied every 3 hours throughout the collection period. Fecal materials were weighed each time the manure catchers were emptied. If fecal materials fell on the ground, these were weighed individually and counted towards the final 24-hour fecal weight but not included in the container containing non-contaminated samples. Upon completion of the 24 hours, total fecal weight was measured in kg. Samples were mixed thoroughly and then a representative sample was taken and placed into a 500 ml plastic jar from Dairy One (Dairy One, Ithaca, NY). Samples were frozen at -20°C until analysis.

If any hay was remaining at the end of the 24-hour collection period, this was collected and weighed in order to determine an accurate 24-hour intake of hay on a kg basis and 24-hour intake of iron (mg, DM basis).

*4.3.3. Sample Analysis*

Serum samples were analyzed for ferritin, serum iron, total iron binding capacity (TIBC), insulin and hepcidin while fecal samples were analyzed for fecal iron.

*4.3.3 a. Serum Ferritin, Iron, Total Iron Binding Capacity (TIBC)*

Analysis for serum ferritin was completed at the Kansas State Veterinary Medical Diagnostic Laboratory (KVMDL, Manhattan, KS). Serum ferritin was measured in ng/ml, with KVMDL noting their adult reference range for horses is 43-261 ng/ml based upon testing data and previous research done by Smith et al. (1984), which noted in their original study a reference range of 70-250 ng/ml (Smith et al., 1984).

Serum iron and TIBC analysis was completed at the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) and measured by automated analyzer. Reference ranges provided by TVMDL were 73-140 ug/dl and 306-518 ug/dl for serum iron and TIBC, respectively.

*4.3.3 b. Hematocrit and Hemoglobin*

Hematology cell counts were analyzed at the end of the Hay and Supplement Phase, with jugular venous samples being collected into vacutainer EDTA tubes (BD Company, Franklin Lakes, NJ). Samples were sent to the North Carolina State Veterinary Hospital Diagnostic Laboratory (Raleigh, NC) for analysis. Hematocrit was measured in percentage while hemoglobin was measured in g/dl and the reference ranges provided by the laboratory were 26.6-44.2% and 10.1-16.4 g/dl, respectively.

*4.3.3 c. Hepcidin*

Hepcidin concentrations were measured in serum samples. A Horse Hepcidin-Compete Enzyme Linked Immunosorbent Assay (ELISA) kit not yet available for commercial use was

used (Intrinsic LifeSciences LLC, La Jolla, CA). The kit was validated by the Pratt-Phillips laboratory at North Carolina State University by dilutional parallelism between kit standards and recovery. All samples were diluted 10% based on kit instructions and run in duplicate. Samples were repeated on subsequent runs if CV >15%.

*4.3.3 d. Insulin*

Insulin concentrations were measured in serum samples for all OSTs. A commercial Human-Insulin Specific Radioimmunoassay kit was used (Kit #HI-14K, EMD Millipore Corporation, Billerica, MA). The sensitivity of the assay is 2.72 μU/ml per the manufacturer. This kit has been previously validated for use in horses (Spears et al., 2020) but was also validated by the Pratt-Phillips laboratory at North Carolina State University. Validation was done by dilutional parallelism between kit standards and linearity in equine serum previously assessed for insulin. All samples were run in duplicate and repeated on subsequent runs if CV >15%.

*4.3.3 e. Fecal Collection*

Fecal samples from the Hay and Supplement Phase were sent to Dairy One (Dairy One, Ithaca, NY) for manure analysis of iron on a ppm basis, in which fecal excretion of iron (mg, DM basis) during the 24-hour fecal collection was determined.

*4.3.4. Statistical Analysis*

Statistical analysis was performed in GraphPad Prism Version 10.2.0 (GraphPad Software, Boston, MA). Descriptive statistics (mean, standard deviation) were calculated, with values presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) for repeated measures was performed on the different variables assessed across the two treatment groups (denoted as IRON, CTRL) and time (denoted as the two study phases: Hay Phase, Supplement Phase). Additionally, multiple comparisons were utilized as needed. Correlation

analysis using Pearson's correlation was performed on variables (e.g., ferritin, hematocrit, hemoglobin) with the association between variables determined based on the correlation coefficient and P-value, with a significance level of $P \leq 0.05$. A trend was noted if $0.05 < P \leq 0.1$. Simple linear regression was also performed to assess the relationship between 24-hour iron intake and fecal excretion of iron.

## 4.4. Results

### *4.4.1. Dietary Intake*

During the Hay Phase, all horses were receiving the same hay and iron-free vitamin mineral supplement. Daily hay intake on a BW basis during this phase ranged from 1.6-2.1%. The reason there was a range in the percentage of daily hay intake was to ensure horses maintained their body weight while minimizing feed refusals. Daily hay intake on a DM basis was 8.98±1.43 kg, with horses consuming 481.64±76.95 mg of iron daily. The NRC recommends daily iron intake to be 400 mg for a 500 kg horse at maintenance (NRC, 2007). Therefore, during this initial study phase, horses were consuming iron similar to NRC recommendations (400 mg for a 500 kg horse) at 481.64±76.95 mg daily.

During the Supplement Phase, all horses still received the same hay and iron-free vitamin mineral supplement, however iron horses consumed an additional daily iron supplement in the form of ferrous sulfate. Daily hay intake on a BW basis during this phase ranged from 1.9-2.4%, and again the reason there was this range was to ensure horses maintained their body weight while minimizing feed refusals. Daily hay intake on a DM basis was 9.997±1.429 kg. Control horses consumed a total of 769.95±49.95 mg iron (DM basis) daily while iron-supplemented horses consumed 3942.99±424.35 mg iron (DM basis) daily when considering both the iron from the hay as well as the supplement. As ferrous sulfate is only 20% elemental iron, the ferrous

sulfate dose ranged from 12,000-19,000 mg, with an average of 3213±405 mg iron provided via the supplement. Therefore, the excess iron was primarily provided via the oral supplement and iron horses were consuming about 10 times the daily NRC iron requirement (NRC, 2007).

*4.4.2. Iron Metabolism Parameters*

Mean ± standard deviation (SD) values of serum ferritin, serum iron, TIBC, hematocrit, hemoglobin and hepcidin are presented in Table 4.4 with reference values presented in Table 4.5.

When looking at serum ferritin, of general interest is the overall high values compared to published reference ranges, which is 43-261 ng/ml (Smith et al., 1984). The majority of horses (10/12; 83.3%) had ferritin values above the reference range during the Hay Phase while 100% did during the Supplement Phase, although IRON horses had higher values compared to CTRL horses (Table 4.4). Analysis of variance noted a trend for a time x treatment interaction (P=0.08) when assessing serum ferritin, in which ferritin values increased in IRON horses (Figure 4.2). Additionally, a paired t-test revealed significant differences (P=0.02) for ferritin between the two phases in horses supplemented with iron.

There were no significant differences in serum iron values between the two phases (P=0.4) and two groups (P=0.9) (Figure 4.3). However, it is important to note that serum iron levels were above reference ranges, especially in IRON horses (Table 4.4, 4.5). Additionally, TIBC values across the two groups of horses and two phases were relatively similar with no significant differences found for a phase x group interaction (P=0.6).

Hematocrit and hemoglobin values are presented in Table 4.4 with reference ranges presented in Table 4.5. There was no significant effect of iron supplementation, however hematocrit and hemoglobin values decreased over time (P<0.05) but were still within the reference range (Figure 4.4). Interestingly, when comparing ferritin with hematocrit and

hemoglobin values across all horses, there was a moderate but significant relationship between ferritin and hemoglobin (Pearson r=0.7; P=0.01) and hematocrit (Pearson r=0.7; P=0.01) following the Hay Phase but there was no relationship following iron supplementation (Hemoglobin: Pearson r=0.5; P=0.08, Hematocrit: Pearson r=0.5; P=0.08).

Hepcidin values are presented in Table 4.4, with ranges of 23.9 - 82.09 ng/ml being noted. When using ANOVA, there was no significant differences between hepcidin values across the different groups and study phases.

*4.4.3. Oral Sugar Test (OSTs)*

Mean ± standard deviation (SD) values of glucose and insulin during both oral sugar tests are presented in Table 4.6 and 4.7 as well as visually in Figure 4.5 and 4.6. There was a significant time effect (P<0.05) for both glucose and insulin during all OSTs (Figure 4.5, 4.6). IRON horses had a significantly higher insulin response (P<0.0001) during the Hay Phase OST (Figure 4.5). During the Supplement Phase OST, there was a time x treatment interaction (P=0.1) trend noted for insulin. When using multiple comparisons, insulin responses were higher (P=0.04) in iron versus control horses at 60 minutes (Figure 4.7). Additionally, insulin responses were higher (P<0.0007) in iron supplemented horses at 0 versus 60 minutes (Table 4.7). Finally, when comparing 60-min insulin values to ferritin concentrations in iron-supplemented horses, an unpaired t-test found this to be significant (P<0.0001).

*4.4.4. Fecal Excretion of Iron*

For fecal excretion of iron over a 24-hour period, a phase x group interaction was found to be significant (P=0.05). Additionally, when utilizing multiple comparisons, fecal excretion of iron was significantly higher (P=0.002) in IRON horses between the Hay and Supplement Phase (Figure 4.8). When assessing the relationship between 24-hour iron intake on a DM basis and 24-

hour fecal iron excretion, there was no significant correlation found between these two variables during both the Hay Phase (P=0.4) while a trend was noted during the Supplement Phase (P=0.1) (Figure 4.9).

**4.5. Discussion**

The results of this study indicate that iron supplementation via an inorganic form of iron influences body iron stores (e.g., ferritin) as well as insulin response to an oral glucose dose. Additionally, iron supplementation does seem to result in increased fecal excretion of iron. Ferritin values were higher in horses supplemented with iron, in which values were 522.5±189.48 ng/ml while control horses had lower ferritin at 460±107.96 ng/ml. However, it is important to note that the majority of horses across the study had ferritin values, an indicator of body iron stores, higher than published reference ranges, which are 43-261 ng/ml (Smith et al., 1984). This reference range was established nearly 40 years ago; thus, it seems that it likely needs to be updated.

The present study yielded similar results to another study, in which ponies were supplemented with ferrous sulfate for 8 weeks to assess the effects of excessive iron on potential adverse clinical effects (Pearson & Andreasen, 2001). Results showed an increase in serum iron and ferritin concentrations due to supplementation, however there were no adverse clinical effects seen in ponies fed excess iron (Pearson & Andreasen, 2001). Similarly, to the Pearson and Andreasen study, a study feeding male pigs excessive iron in the form of ferrous sulfate for 60 days resulted in an increase in serum iron, although this was not found to be statistically significant (Middleton et al., 2021). There was no significant difference in serum iron concentrations in horses supplemented with iron during the present study, with these values being 176.9±34.93 and 237.2±150.74 ug/dl, during the Hay and Supplement Phase, respectively,

however these values were over the laboratory's provided reference range (73 – 140 ug/dl). Similarly, Lawrence et al. (1987) failed to see an increase in serum iron concentrations despite supplementing ponies with iron (Lawrence et al., 1987), thus it appears that results are varied across different studies and/or species.

Results from the present study indicated a significant decrease in hematocrit and hemoglobin despite horses being supplemented with iron, however these values were still within reference range. Hemoglobin is a protein within red blood cells that carries oxygen and is where the majority of body iron is found (Dev & Babitt, 2017) while hematocrit is the percentage of red blood cells within the body. Both Pearson and Andreasen and Lawrence et al. (1987) failed to see a change in hemoglobin and/or hematocrit in ponies despite iron supplementation (Lawrence et al., 1987; Pearson & Andreasen, 2001). However, a study with pigs supplemented with excess iron saw a more substantial increase in hematocrit and hemoglobin values versus pigs fed a control diet (Middleton et al., 2021). Therefore, it appears that iron supplementation has mixed results on hematocrit and hemoglobin, however this is likely due to the tight regulation of iron within the body as well as erythropoiesis occurring in the bone marrow (Yiannikourides & Latunde-Dada, 2019).

Hepcidin is a peptide hormone and thought to be the master regulator of systemic iron homeostasis, in which hepcidin expression will be modified based on various factors like iron overload, anemia, iron deficiency, body iron status and inflammation (Camaschella et al., 2020; Dev & Babitt, 2017; Grimes et al., 2012). Data on hepcidin is limited in horses, although hepcidin has been identified in horses (Oliveira Filho et al., 2010) and its expression has been found to be upregulated in times of inflammation (Oliveira-Filho et al., 2012; Oliveira-Filho et al., 2014). Studies that have evaluated serum hepcidin are limited, in which a recent study of

captive Mongolian horses found hepcidin values of 110±40 ng/ml (Wijekoon et al., 2022) while the present study reported serum hepcidin ranges of 24 - 82 ng/ml. Additionally, pregnant mares had decreased hepcidin concentrations throughout gestation, with values at 158 ± 7 ug/dl in mares at 1 month versus values of 63 ± 26 ug/dl in mares at 11 months of pregnancy (Satué et al., 2023). Based on the limited studies done, measuring serum hepcidin may be useful to assess iron status in horses, especially when addressing inflammatory issues.

Oral sugar tests (OST) were performed to assess the effects of iron supplementation on glucose metabolism. A dose of 0.45 ml/kg of BW of Karo syrup was utilized for the present study per recommendations by the Equine Endocrinology Group as this dose has been shown to improve test performance (Jocelyn et al., 2018). Additionally, a study that utilized the 0.45 ml/kg dose for an OST was able to identify more incidences of insulin dysregulation compared to basal insulin (Box et al., 2020; Van Den Wollenberg et al., 2020). However, a recent study by Macon et al. (2021) saw no differences in mean insulin concentrations between insulin-dysregulated and non-insulin-dysregulated horses when using a low dose of Karo Syrup (0.15 ml/kg BW) versus a high dose (0.45 ml/kg BW), indicating more work needs to be done when to discern a higher dose for OSTs (Macon et al., 2021). Although none of the insulin values across either group in the present study were >65 μU/ml, which is indicative of insulin dysregulation based on the 0.45 ml/kg BW dose and recommendations from the Equine Endocrinology Group (Frank et al., 2022), insulin responses were higher in iron-supplemented horses when compared to controls after 60 minutes, indicating a possible positive correlation between iron and insulin response.

There have been some studies in horses that have suggested a potential correlation between iron and insulin resistance (Kellon & Gustafson, 2019; Nielsen et al., 2012). In a study by Nielsen et al. (2012), a correlation between elevated ferritin and higher insulin response after

dextrose administered was determined, thus indicating a potential correlation between these (Nielsen et al., 2012). Additionally, Kellon and Gustafason (2019) found a correlation between elevated ferritin and horses considered hyperinsulinemic (Kellon & Gustafson, 2019). In humans, serum ferritin has been found to be associated with excessive body fat, which could lead to metabolic issues like insulin resistance (Moore Heslin et al., 2021). Therefore, it does seem plausible that body iron status, insulin response and metabolic and/or inflammatory issues (e.g., obesity) could be interrelated.

Fecal collections occurred during the study in order to assess fecal excretion of iron over a 24-hour period. Iron excretion was significantly higher in iron supplemented horses between the Hay (104 ± 83 mg) and Supplement Phase (248 ± 168 mg). Iron horses received a total of 3942.9±424.35 mg of iron daily, provided from both the hay and iron supplement. Ferrous sulfate is considered to be highly bioavailable, with various studies across different species finding relative bioavailability to be 100% (Henry & Miller, 1995). Thus, it seems plausible that the high bioavailability of the ferrous sulfate consumed by the iron-supplemented horses resulted in more fecal excretion of iron.

Studies assessing iron digestibility are limited in horses, as it is difficult to determine true digestibility, which accounts for endogenous losses and this has not been reported in horses (NRC, 2007). One study evaluated three different types of hay to determine digestibility and apparent mineral absorption where fecal collections occurred over 5 days. Fecal excretion across the 3 different types of hay ranged from 1.4 - 4 grams/day, with apparent absorption ranging from -0.5 to -0.9 g/d (Crozier et al., 1997). Based on the NRC, the hay from this study exceeded the 40 ppm recommendations but negative absorption was seen (Crozier et al., 1997). However, the reason for a higher fecal excretion versus intake could be due to sloughing and desquamation

of epithelial cells and blood loss (Dev & Babitt, 2017), and this could be a factor in the present study seeing an increase in fecal iron excretion, especially in horses supplemented with iron. Additionally, as there is no active excretion mechanism for iron (Andrews & Schmidt, 2007), this may be playing a role as well. Another study evaluated iron metabolism in exercising horses over a 3-week exercise period where fecal, urine and sweat samples were collected (Inoue et al., 2005). Excretion of iron via the urine was less than 1% of apparent absorption but iron was lost via sweat during the exercise periods, however this was less than 5% of apparent absorption. However, iron absorption was increased throughout the exercise periods, likely due to hemolysis (Inoue et al., 2005).

Despite the present study seeing relationships between dietary iron with supplementation and iron and glucose metabolism parameters as well as digestibility, there were some limitations in the current study. First, supplementation of iron was done with an inorganic source of iron through ferrous sulfate. Although ferrous sulfate is highly bioavailable (Henry & Miller, 1995), it only contains 20% elemental iron, and is likely not the form of iron horses would be consuming on a forage-only diet. Thus, future research should assess feeds naturally high in iron to determine if there are any differences in iron and glucose metabolism parameters based on the different forms of iron. Second, fecal collections were only performed for 24-hours due to time and equipment constraints. Higher apparent digestibility of iron seen in iron-supplemented horses could have been due to increased sloughing of cells and blood loss, resulting in higher-than-normal digestibility. Therefore, future studies should utilize a longer collection period. Despite the limitations, the present study does indicate there are relationships among body iron status, dietary iron intake and glucose metabolism that should be explored further.

## 4.6. Conclusion

The present study saw an increase in ferritin concentrations in horses supplemented with iron. Although iron-supplemented horses were not classified as having insulin dysregulation, insulin responses were higher in iron-supplemented horses versus controls after an oral sugar test. Thus, it seems that there is a correlation between elevated body iron stores and insulin responses. However, this should be explored further, in particular utilizing feeds naturally high in iron. Finally, supplementation of iron via ferrous sulfate results in an increased excretion of iron via the feces.

## Tables and Figures

**Table 4.1.** Complete nutrient analysis of orchardgrass-timothy hay used during the first 28 days of the study (Hay Phase) completed by the North Carolina Department of Agriculture and Consumer Services

| Analyte | Content[a] |
|---|---|
| Dry Matter (%) | 89.80 |
| Crude Protein (%) | 8.48 |
| Digestible Energy (mcal/lb) | 0.91 |
| Calcium (%) | 0.37 |
| Phosphorus (%) | 0.17 |
| Sodium (%) | 0.02 |
| Copper (ppm) | 4.90 |
| Iron (ppm) | 53.63 |
| Zinc (ppm) | 12.34 |
| Ash (%) | 6.72 |

[a]Nutrient values are presented on a 100% dry matter basis

**Table 4.2.** Guaranteed analysis of SmartVite Thrive Pellets (Smart Pak Equine, Plymouth, MA) per 1 scoop (40 g) using during the study

| Analyte | Content |
|---|---|
| Crude Protein (%) | 5% |
| Calcium (%) | 1.75 (min), 2.25 (max) |
| Phosphorus (%) | 1.1 |
| Salt (%) | 2.5 (min), 2.6 (max) |
| Magnesium (mg) | 1500 |
| Potassium (mg) | 450 |
| Copper (ppm) | 312.5 |
| Zinc (ppm) | 937.5 |
| Vitamin A (IU) | 7500 |
| Vitamin D3 (IU) | 750 |
| Vitamin E (IU) | 125 |

Ingredients: Dehydrated Alfalfa Meal, Corn Distillers Dried Grains with Solubles, Magnesium Oxide, Monocalcium Phosphate, Calcium Carbonate, Salt, Lignin Sulfonate, Monosodium Phosphate, Heat Stabilized Rice Bran, Vegetable Oil, Flaxseed Meal, Selenium Yeast, Dried Kelp, Vitamin E Supplement, Calcium Propionate (preservative), Zinc Polysaccharide Complex, Zinc Sulfate, Manganese Polysaccharide Complex, Manganese Sulfate, Ascorbic Acid, Copper Polysaccharide Complex, Copper Sulfate, Biotin, Zinc Proteinate, Manganese Proteinate, Copper Proteinate, Thiamine Mononitrate, Niacin, Riboflavin, Vitamin A Acetate, Natural & Artificial Flavors, Menadione Sodium Bisulfite Complex (source of Vitamin K activity), Pyridoxine Hydrochloride, d-Calcium Pantothenate, Folic Acid, Beta Carotene, Vitamin B 12 Supplement,

Ethylenediamine Dihydriodide, Vitamin D3 Supplement, Coconut Oil, Cobalt Carbonate, Sodium Propionate (preservative), Citric Acid (preservative)

**Table 4.3.** Complete nutrient analysis of orchardgrass-timothy hay used during the latter 28 days of the study (Supplement Phase) completed by the North Carolina Department of Agriculture and Consumer Services

| **Analyte** | **Content[a]** |
|---|---|
| Dry Matter (%) | 90.42 |
| Crude Protein (%) | 8.83 |
| Digestible Energy (mcal/lb) | 1.01 |
| Calcium (%) | 0.34 |
| Phosphorus (%) | 0.08 |
| Sodium (%) | - |
| Copper (ppm) | 4.27 |
| Iron (ppm) | 74.11 |
| Zinc (ppm) | 14.99 |
| Ash (%) | 5.20 |

[a]Nutrient values are presented on a 100% dry matter basis

**Figure 4.1.** Bun-Bag Manure Catcher fitted to each horse and used during the 24-hour total fecal collection

**Table 4.4.** Serum values of ferritin, serum iron, TIBC, hematocrit, hemoglobin and hepcidin measured across IRON and CONTROL horses during the two study phases (Hay Phase, Supplement Phase). All values are presented as mean ± standard deviation (SD)

| | **Hay Phase** | | **Supplement Phase** | |
|---|---|---|---|---|
| **Variable** | **Iron** | **Control** | **Iron** | **Control** |
| Ferritin (ng/ml) | 428.9±174.20 | 479.3±168.21 | 522.5±189.48 | 460.0±107.96 |
| Serum Iron (ug/dl) | 176.9±34.93 | 172.3±17.32 | 237.2±150.74 | 187.5±26.83 |
| TIBC (ug/dl) | 366.8±30.03 | 361.8±26.69 | 463.7±261.20 | 389.9±64.49 |
| Hematocrit (%) | 37.8±3.49 | 40.0±3.59 | 36.3±3.89 | 35.9±4.78 |
| Hemoglobin (g/dl) | 13.1±1.19 | 13.5±1.23 | 12.6±1.34 | 12.5±1.53 |
| Hepcidin (ng/ml) | 46.6±17.81 | 60.8±2.97 | 44.7±18.08 | 57.6±18.17 |

**Table 4.5.** Reference ranges provided by laboratories for variables (ferritin, serum iron, TIBC, hematocrit, hemoglobin and hepcidin) measured throughout the study

| Variable | Reference Range |
|---|---|
| Ferritin (ng/ml) | 43 – 261 |
| Serum Iron (ug/dl) | 73 - 140 |
| TIBC (ug/dl) | 306 - 518 |
| Hematocrit (%) | 26.6 – 44.2 |
| Hemoglobin (g/dl) | 10.1 – 16.4 |
| Hepcidin (ng/ml) | Unknown |

**Figure 4.2.** Ferritin values (measured in ng/ml) in control (denoted in blue) and iron (denoted in red) horses across the two study phases (Hay and Supplement Phase). The significant difference in ferritin values seen between the Hay and Supplement Phase in IRON horses is denoted by the symbol*

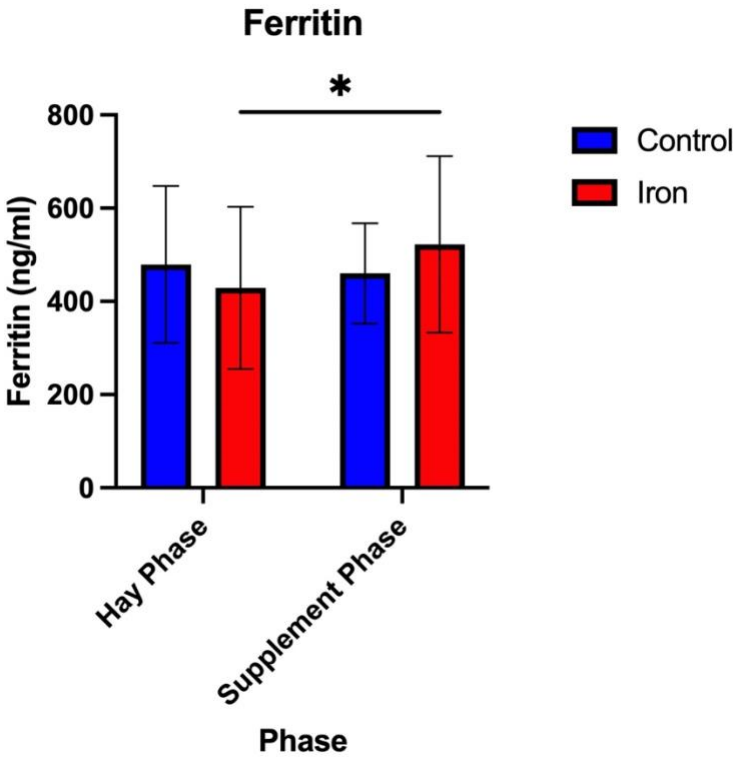


**Figure 4.3.** Serum iron (measured in ug/dl) and TIBC (measured in ug/dl) values in control (denoted in blue) and iron (denoted in red) horses across the two study phases (Hay and Supplement Phase). No significant differences were found between groups, phases or a phase x group interaction for both serum iron and TIBC values

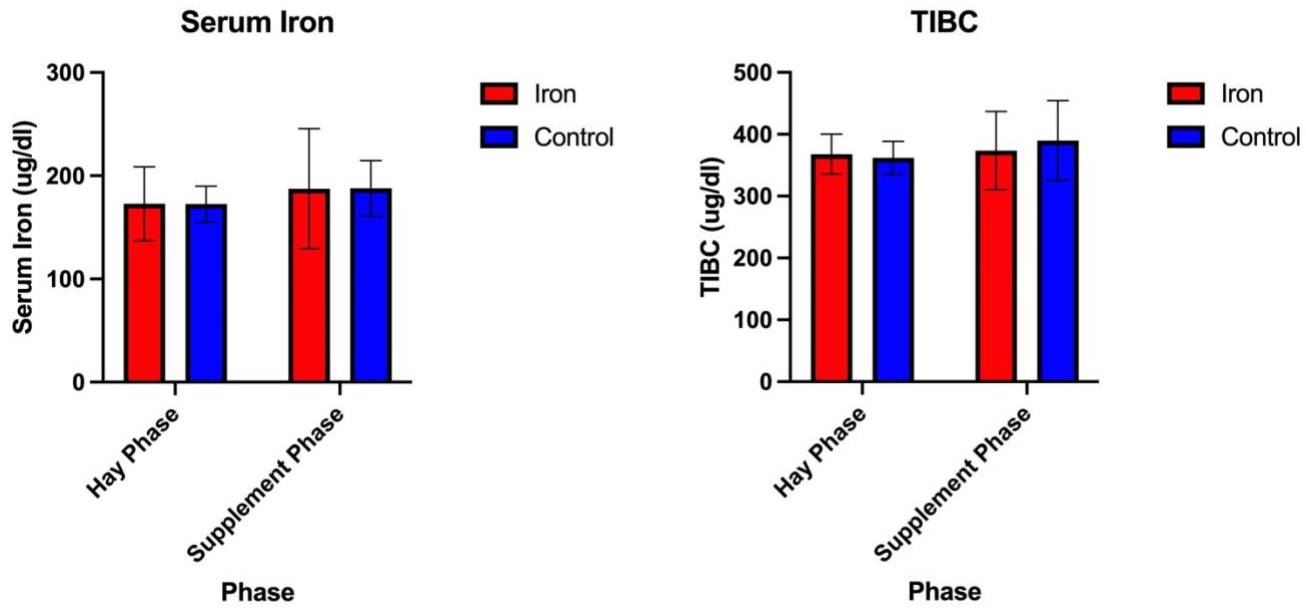


**Figure 4.4.** Hemoglobin (measured in g/dl) and hematocrit (measured in %) values in control (denoted in blue) and iron (denoted in red) horses across the two study phases (Hay and Supplement Phase). There was a significant phase ($P<0.05$) effect but no significant differences seen in iron versus control horses

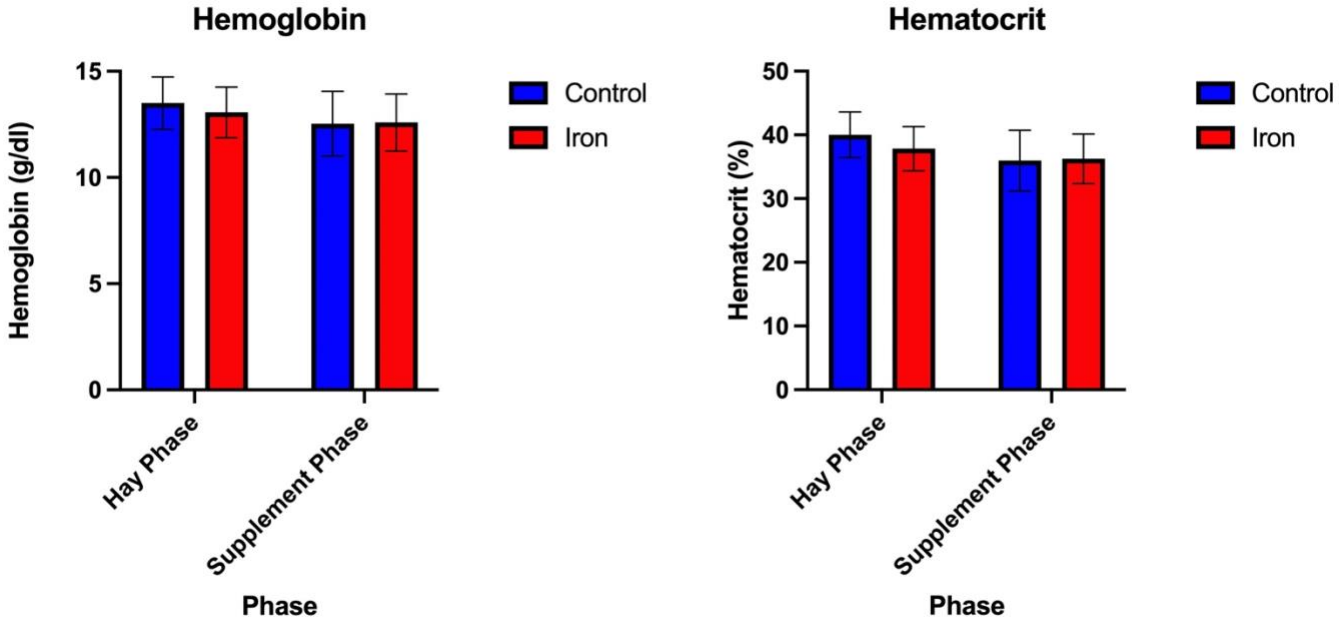


**Figure 4.5.** Glucose (measured in g/dl) and insulin (measured in μU/ml) values in control (denoted in blue) and iron (denoted in red) horses during the Hay Phase OST. There was a significant time effect (P<0.05) for both glucose and insulin

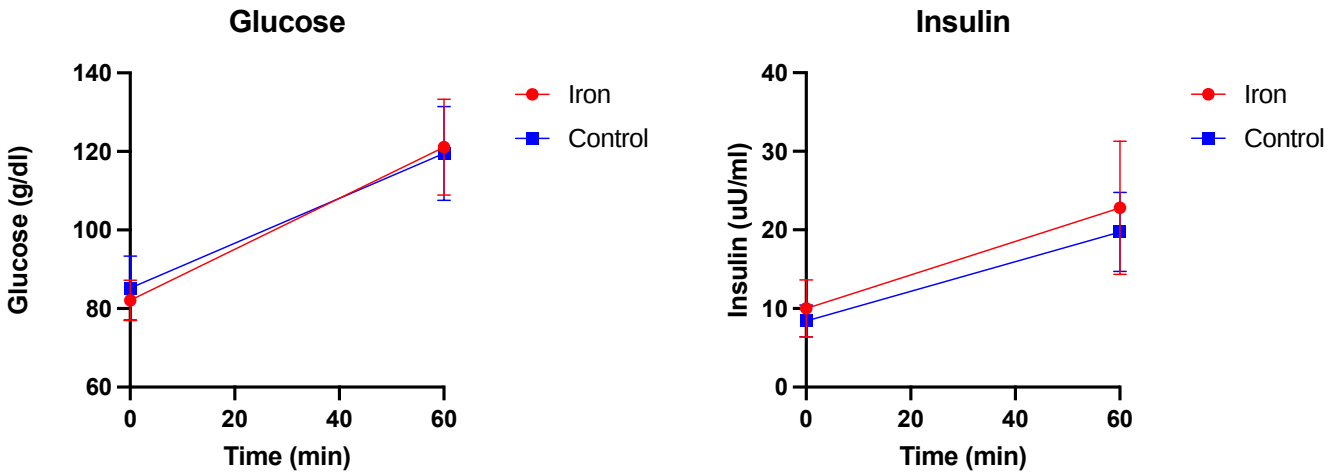



**Table 4.6.** Glucose and insulin values measured at 0 and 60 minutes in iron and control horses during Hay Phase OST. All values are presented as mean ± standard deviation (SD)

| | 0 Min | | 60 Min | |
|---|---|---|---|---|
| **Variable** | **Iron** | **Control** | **Iron** | **Control** |
| Glucose (g/dl) | 82.1±5.14 | 85.3±8.18 | 121.1±12.22 | 119.5±11.90 |
| Insulin (μU/ml) | 10.0±6.65 | 8.4±2.05 | 22.8±8.48 | 19.7±5.05 |

**Figure 4.6.** Glucose (measured in g/dl) and insulin (measured in μU/ml) values in control (denoted in blue) and iron (denoted in red) horses during the Supplement Phase OST. There was a significant time effect (P<0.05) for both glucose and insulin

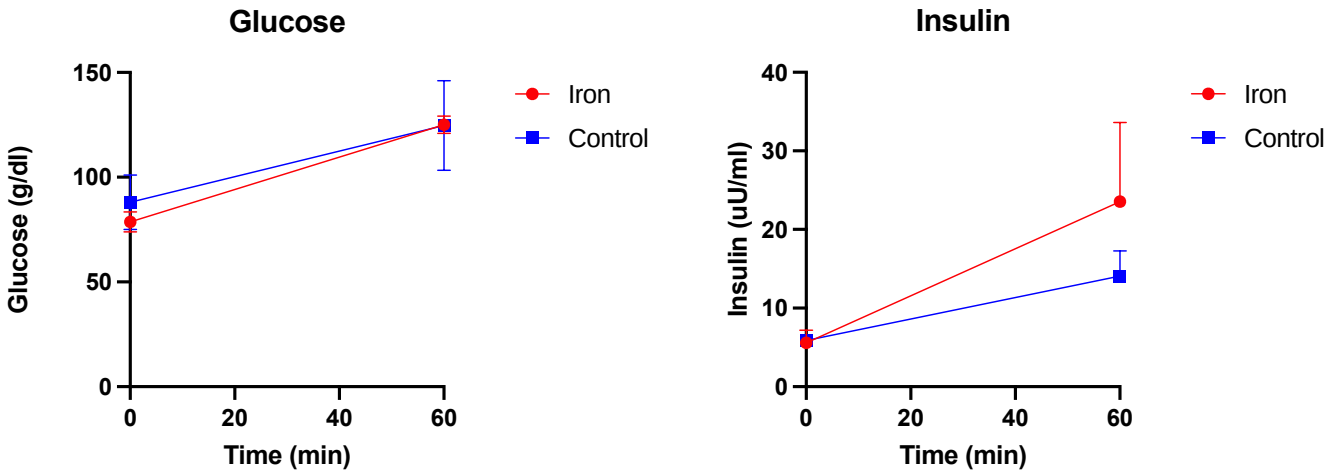



**Table 4.7.** Glucose and insulin values measured at 0 and 60 minutes in iron and control horses during Supplement Phase OST. All values are presented as mean ± standard deviation (SD)

| | 0 Min | | 60 Min | |
|---|---|---|---|---|
| **Variable** | **Iron** | **Control** | **Iron** | **Control** |
| Glucose (g/dl) | 78.7±4.76 | 88.0±13.00 | 125.0±4.15 | 124.7±21.36 |
| Insulin (μU/ml) | 5.6±1.60 | 5.9±0.28 | 23.6±10.05 | 14.1±3.21 |

**Figure 4.7.** Insulin (measured in μU/ml) values in control (denoted in blue) and iron (denoted in red) horses during the Supplement Phase OST. Significant differences are indicated by the different letters[a,b], in which there was a higher insulin response (P=0.04) in iron-supplemented horses compared to control horses at 60 minutes

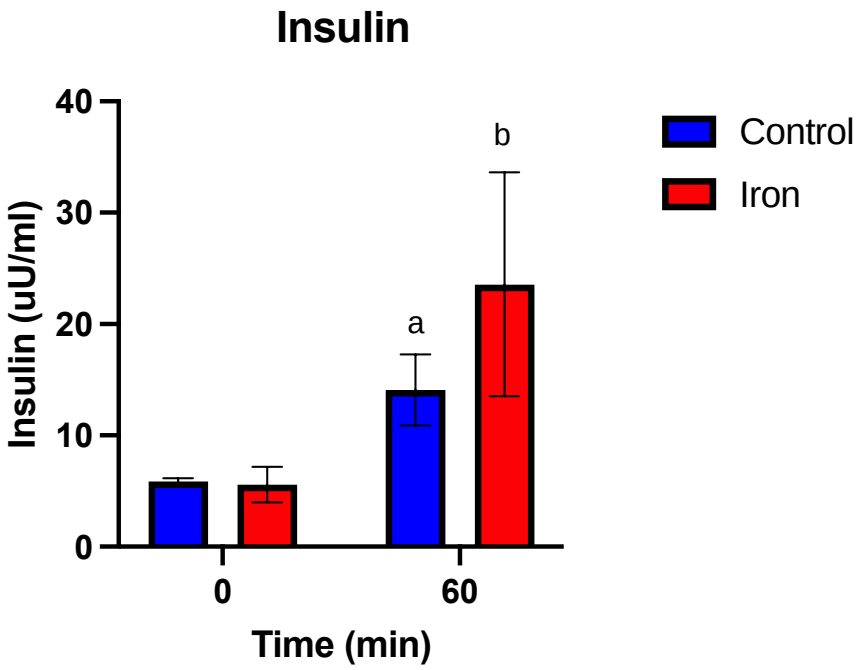


**Figure 4.8.** Fecal iron excretion (measured in mg, DM basis), in control (denoted in blue) and iron (denoted in red) horses measured over a 24-hour period during the Hay Phase and Supplement Phase. Significant differences are indicated by the different letters[a,b], in which there was higher fecal iron excretion (P=0.002) in iron-supplemented horses during the Supplement Phase compared to the Hay Phase

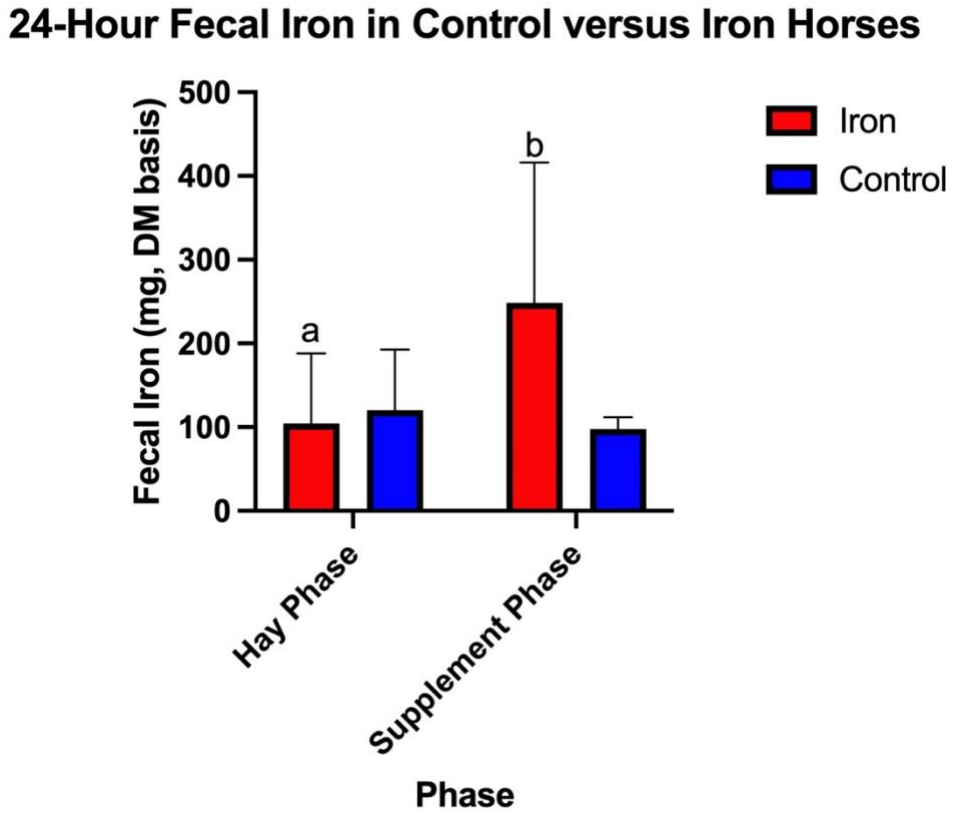
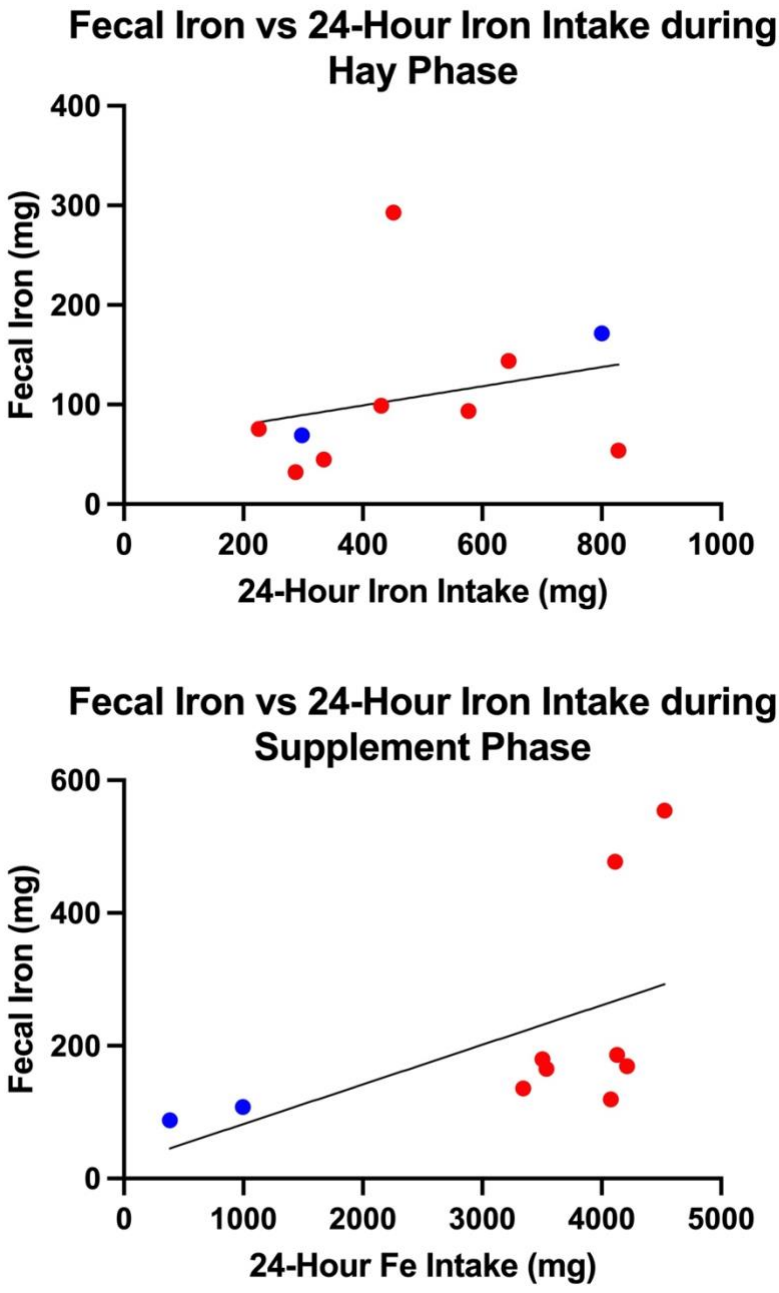


**Figure 4.9.** Simple linear regression plot of 24-hour iron intake (measured in mg, DM basis, denoted on the x-axis) and 24-hour fecal iron excretion (measured in mg, denoted on the y-axis) during the Hay Phase (top) and Supplement Phase (bottom) with control horses denoted in blue and iron horses in red. There was no significant correlation between these variables during the Hay Phase but a trend (P=0.1) was noted between these during the Supplement Phase



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# CHAPTER 5: STUDY 4

## Utilizing an iron absorption test in horses: a pilot study

### 5.1. Abstract

Oral iron absorption tests (IATs) have been performed regularly in humans to evaluate iron absorption and potential iron related disorders like iron-deficiency anemia. However, this type of testing has not been documented in horses. Thus, the objective of this pilot study was to perform an oral iron absorption test before and after iron supplementation to assess if this affected iron metabolism parameters (e.g., serum ferritin) responses to a large dose of iron. Mixed-breed geldings (n=12) were used and housed individually throughout the study, consuming 2% of their body weight in mixed-grass hay and an iron-free vitamin-mineral supplement. On day 5, the first IAT was performed (denoted as Pre), in which horses received 25 mg/kg of BW of elemental iron in the form of ferrous sulfate orally. Jugular venous samples were taken before administration of the iron dose and subsequent samples were taken 3-, 6-, 12- and 24-hours post dose. Samples were centrifuged and serum was aliquoted then frozen at -20°C until analysis. After the first 28 days of the study, horses were randomly assigned to two groups: IRON (n=8) or CTRL (n=4), in which IRON horses were supplemented with an oral iron supplement, consuming an average of 4000 mg Fe daily on a DM basis while CTRL horses remained on the same hay diet. At the end of the study, another IAT was performed (denoted as Post), following the procedures outlined above. Serum samples were analyzed for serum ferritin, iron, total iron binding capacity (TIBC) and hepcidin. Statistical analysis was performed in both GraphPad Prism Version 10.2.0 (GraphPad Software, Boston, MA) and SAS Studio (SAS Institute, Inc., Cary, NC), utilizing analysis of variance as well as calculating area under the curve (AUC). There was a phase (Pre, Post) x treatment (IRON, CTRL) interaction (P=0.002) for

serum ferritin, in which ferritin values increased for IRON horses. Interestingly, serum iron concentrations peaked at 3 hours during both IATs while serum ferritin levels were lowest at this time point. When calculating AUC for serum hepcidin during the Post phase IAT, serum hepcidin responses were higher (P=0.01) in iron-supplemented horses compared to controls. The results indicate that supplementation with an inorganic form of iron seems to result in increased responses of ferritin and hepcidin. Further research needs to be done using varying sources of iron as well as doses and iron absorption tests may be beneficial to evaluate iron status in horses.

**5.2. Introduction**

Iron is a micromineral involved in several essential physiological functions throughout the body such as oxygen transport and oxidative phosphorylation (Andrews & Schmidt, 2007; Dev & Babitt, 2017). Iron homeostasis is tightly regulated in order to maintain essential processes such as erythropoiesis, which occurs in the bone marrow and is therefore the largest consumer of iron within the body (Yiannikourides & Latunde-Dada, 2019). Iron homeostasis must also be tightly maintained due to iron having no active excretion mechanism from the body, therefore total body iron is regulated from the point of absorption. One of the key players of systemic iron homeostasis is hepcidin, a peptide hormone produced in the liver by the hepatocytes and is highly conserved across different species (Camaschella et al., 2020; Grimes et al., 2012). Hepcidin was discovered in the early 2000s, and the discovery of this acute phase protein was a breakthrough in understanding how iron is regulated systemically and its relation to iron disorders, such as iron overload and anemia (Dev & Babitt, 2017; Grimes et al., 2012; Yiannikourides & Latunde-Dada, 2019).

Hepcidin research is still limited in horses, however since its discovery it has been sequenced and identified in horses and has been shown to be upregulated during times of

inflammation, which is similar to humans (Grimes et al., 2012; Oliveira Filho et al., 2010; Oliveira-Filho et al., 2012; Oliveira-Filho et al., 2014). Research on serum hepcidin levels in normal horses is extremely limited due to a lack of commercially available analytical methods, however in a human study comparing patients on dialysis to control patients, serum hepcidin levels were higher in hemodialysis patients compared to controls, although this was not found to be significant (Taheri et al., 2015). As hepcidin has been known to be associated with inflammation, inflammatory disorders may play a role in an increase of serum hepcidin levels (Andrews & Schmidt, 2007; Dev & Babitt, 2017). Published serum hepcidin levels are limited in horses, although a study of captive Mongolian horses found serum hepcidin to be 110.±40 ng/ml (Wijekoon et al., 2022) while another study found that serum hepcidin levels in mares decreased over 11 months of pregnancy (Satué et al., 2023).

When iron is stored in the body, it is generally stored as ferritin, a storage protein found in the hepatocytes of the liver in order to minimize circulating iron (Roemhild et al., 2021). Ferritin can store up to 4500 atoms of iron and by being stored as this protein, it can mediate systemic iron delivery into the bloodstream by allowing more regulated control of iron to basolateral exporters (e.g., ferroportin) (Camaschella et al., 2020; Dev & Babitt, 2017; Donovan et al., 2000). Although ferritin is in a constant state of turnover due to its short lifespan (Kamerling & Tobin), serum ferritin is thought to be the most convenient way to measure body iron status in humans and horses (Abbaspour et al., 2014; NRC, 2007; Smith et al., 1986; Smith et al., 1984). Smith et al. (1984) found a range of 70-250 ng/ml of serum ferritin in mature, adult horses (Smith et al., 1984) and this reference range is primarily used today at diagnostic laboratories. However, a study utilizing horses as a model for black rhinoceros to investigate potential correlations between iron overload and insulin resistance found elevated serum ferritin

and peak insulin to be positively correlated (Nielsen et al., 2012). Additionally, this study noted serum ferritin levels ranged from 130-882 ng/ml, thus some of these levels being above the reference range set by Smith et al. (1984) (Nielsen et al., 2012; Smith et al., 1984).

Oral iron absorption tests (IAT) have been frequently used in humans to evaluate intestinal iron absorption (Andersen et al., 2015). Iron-deficiency anemia is considered one of the most common nutritional deficiencies worldwide in humans (Kumar et al., 2022) and is classified by insufficient iron intake, decreased absorption of iron and ultimately an increase in iron loss (NILSSON-EHLE et al., 1988, 1989; Patel, 2008; Rondinelli et al., 2017). In order to reverse iron-deficiency anemia, oral iron has been given therapeutically in humans. Therefore, IATs are useful to assess anemic patients (as well as others) to evaluate how they absorb iron by measuring plasma and/or serum iron concentrations after being given an oral iron dose (Andersen et al., 2015). Markers of iron such as serum iron, ferritin and hepcidin have been measured in human studies using IATs, in which peak serum iron and ferritin have been found to be inversely correlated after an oral iron dose of ferrous sulfate (Joosten et al., 1997), and patients with anemia associated with gastrointestinal abnormalities had low serum hepcidin levels during an IAT (Loveikytea et al., 2023). Although there have been a number of studies done in humans, there is no commonly used protocol for this absorption test, with research noting a variety of types of iron as well as doses (Jensen et al., 1998). However, based on the literature, it does appear that oral iron absorption tests in humans are a beneficial diagnostic tool to assess iron absorption within the body and potentially diagnose iron and/or inflammatory related disorders, such as iron-deficiency anemia.

Iron deficiency is not as common in horses compared to humans (Geor et al., 2013; NRC, 2007). Generally, milk-fed foals are most susceptible to hypochromic anemia but this is not

common in older foals or mature horses as long as they have access to soil (NRC, 2007). On the opposite spectrum, iron toxicity is generally not considered an issue in horses, although in mature horses ferrous fumarate toxicity has been reported (Arnbjerg, 1981; NRC, 2007). Additionally, it has been suggested that serum iron concentrations above 400 ug/dl could indicate acute toxicosis (Puls, 1994), but serum iron can be affect by a number of different issues. A horse's daily iron requirement is generally met through access to a forage-based diet and/or soil, therefore supplementation is not necessary, although it is common in exercising and/or competition horses due to perceived performance benefits (Geor et al., 2013; McLean et al., 2022).

To the authors' knowledge, iron absorption tests have not been done and/or documented in horses before. Therefore, the objective of the study was to perform an iron absorption test to assess if a large dose of iron changed iron metabolism parameters such as serum iron, ferritin, total iron binding capacity and hepcidin. It was hypothesized that an oral dose of iron would increase iron metabolism parameters such as serum ferritin, hepcidin and serum iron.

## 5.3. Materials and Methods

The North Carolina State University Institutional Animal Care and Use committee approved this study (Protocol #23-173-01).

### *5.3.1. Animals*

All horses used for the study were housed at the North Carolina State University Reedy Creek Farm and kept on cool-season mixed pasture for the previous 4 months prior to the study. Additionally, all horses had access to free choice white salt and automated waterers. Horses used in the study were mixed-breed geldings (n=12) with a mean ± standard deviation (SD) body weight (BW) of 546.4±74.43 kg and body condition scores (BCS) ranging from 4.2-7 and a

mean value of 5.7±0.90. The 1-9 scale for Body Condition Score (BCS) developed by Henneke was utilized for the study (Henneke et al., 1983). All horses were weighed using a livestock scale (Gallagher 210 Livestock Scale, Gallagher USA, Riverside, MO) at the beginning of the study. Horses were re-weighed approximately every 7 days during the initial study period and feed adjustments were made as necessary if there were body weight changes.

*5.3.2. Procedure*

Horses were housed in individual, partially-covered, 3.7 x 12.2-meter dry lot pens with automatic waterers and had access to free-choice salt. Water samples were taken, sent to the North Carolina Department of Agriculture and Consumer Services (NCDA&CS, Raleigh, NC) Agronomic Division and was determined to contain 0.04 ppm of iron. Horses were fed an orchardgrass-timothy hay. A representative hay sample was also taken and sent to the NCDA&CS (Raleigh, NC) for complete nutrient analysis (Table 5.1) and contained 53.63 ppm of iron on a dry matter (DM) basis. At the start of the study, horses were offered 2% of their body weight in hay, however refusals were recorded and hay offered was adjusted based on this, thus horses were offered anywhere from 1.5-2% of their body weight in hay throughout the study.

In order to ensure all nutrient requirements were met based on the National Research Council recommendations for mature horses at maintenance (NRC, 2007), an iron-free vitamin mineral supplement, SmartVite Thrive Pellets (SmartPak Equine, Plymouth, MA) was provided at 1 scoop daily, or approximately 40 g (Table 5.2). Additionally, powdered Vitamin E (200 IU), zinc (100 mg) and copper (1500 mg) were provided (Bulk Supplements, Henderson, NV).

The study was divided into two phases, the Hay Phase and Supplement Phase. On day 5 of the study, an iron absorption test (IAT) was performed (denoted as Pre), which is detailed

further below. The first 28 days of the study (Hay Phase) was considered an acclimation period in which all horses were consuming the same diet.

For the latter 28 days of the study (Supplement Phase), horses were randomly assigned into 2 groups, CTRL (Control; n=4) or IRON (Supplement; n=8). Both CTRL and IRON horses consumed 1.5-2% of their body weight in orchardgrass-timothy hay daily. A representative hay sample was taken and sent to the North Carolina Department of Agriculture & Consumer Services (NCDA&CS, Raleigh, NC) for complete nutrient analysis (Table 5.3) and contained 74.11 ppm of iron on a dry matter (DM) basis. The reason the iron content of this hay was slightly higher was due to a different hay supply being utilized for the latter 28 days of the study. Horses in the CTRL group remained on the same hay and vitamin-mineral supplement while IRON horses received an iron supplement. The iron supplement in the form of ferrous sulfate was administered orally daily. The ferrous sulfate was a powdered-form and classified as animal feed grade (Alpha Chemicals, Missouri, USA). As the ferrous sulfate was a powder, it was weighed out and mixed thoroughly with sugar-free applesauce before being administered to the horses in an oral dosing syringe every morning. The iron dose used was 3000 mg/500 kg BW or 6 mg/kg BW. This dose was determined based on previous work done in which ponies administered 50 mg of iron per kg of BW in the form of ferrous sulfate for 8 weeks displayed no adverse clinical effects (Pearson & Andreasen, 2001). As ferrous sulfate only contains 20% elemental iron, this was factored in when calculating the daily dosage. At the end of the Supplement Phase, another IAT was performed (denoted as Post), which is detailed further below.

*5.3.3 Iron Absorption Test (IAT)*

An oral iron absorption test (IAT) was performed to assess the effects of a large dose of iron given orally on iron metabolism parameters (e.g., hepcidin, ferritin) over a 24-hour period. On day 5 of the study, the first IAT was performed (denoted as Pre), with the test beginning at approximately 7:00 AM. Time 0 jugular venous samples were taken in vacutainer serum tubes containing no anticoagulant (BD Company, Franklin Lakes, NJ), collecting approximately 20 ml.

For the iron dose, animal-feed grade ferrous sulfate was utilized (Alpha Chemicals, Missouri, USA). As there is no prior research done to the authors' knowledge in which horses undergo an iron absorption test (IAT), the iron dose was determined based on a study by Pearson and Andreasen (2001) (Pearson & Andreasen, 2001). In humans, previous studies have completed IATs in which doses have ranged from 10 mg of iron sulphate (Jensen et al., 1998), 20 mg (Joosten et al., 1997) and 50 mg of iron in the form of sodium ferrous citrate (Kobune et al., 2011). Based on this prior work, the present study utilized an iron dose of 25 mg/kg of BW of elemental iron. As ferrous sulfate is only 20% elemental iron, this was factored into the iron dose, therefore horses received a ferrous sulfate dose of 125 mg/kg of BW. Individual horse doses were calculated based on body weight and the ferrous sulfate was weighed out. The ferrous sulfate was a powdered form so it was mixed thoroughly with sugar-free applesauce then administered to each horse via an oral dosing syringe (Figure 5.1), ensuring minimal iron dose was spilled. Once horses received their iron dose, they were returned to their dry lot pens and allowed access to feed as normal. Additionally, horses were monitored for any adverse effects to the iron dose. Subsequent jugular venous samples were taken 3-, 6-, 12- and 24-hours after the iron dose was administered and collected via vacutainer serum tubes containing no anticoagulant (BD Company, Franklin Lakes, NJ), collecting approximately 20 ml of blood at each time point.

After the 24-hour jugular venous sample was completed, the IAT was complete. Blood samples collected in serum vacutainer tubes (BD Company, Franklin Lakes, NJ) were allowed to clot then centrifuged at 1200 x *g* at 4°C for approximately 15 minutes. Upon completion of centrifugation, serum was aliquoted into microcentrifuge tubes (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA) and frozen at -20°C until analysis.

The procedure outlined above was performed again at the end of the study for the second IAT (denoted as Post).

*5.3.4. Sample Analysis*

Serum samples were analyzed for serum ferritin, iron, total iron binding capacity (TIBC) and hepcidin.

*5.3.4 a. Serum Ferritin, Iron, Total Iron Binding Capacity (TIBC)*

Analysis for serum ferritin was completed at the Kansas State Veterinary Medical Diagnostic Laboratory (KVMDL, Manhattan, KS). Serum ferritin was measured in ng/ml, with KVMDL noting their adult reference range for horses is 43-261 ng/ml based upon testing data and previous research done by Smith et al. (1984), which noted in their original study a reference range of 70-250 ng/ml (Smith et al., 1984).

Serum iron and TIBC analysis was completed at the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) and measured by automated analyzer. Reference ranges provided by TVMDL were 73-140 ug/dl and 306-518 ug/dl for serum iron and TIBC, respectively.

*5.3.4 b. Hepcidin*

Hepcidin concentrations were measured in serum samples. A Horse Hepcidin-Compete Enzyme Linked Immunosorbent Assay (ELISA) kit not yet available for commercial use was

used (Intrinsic LifeSciences LLC, La Jolla, CA). The kit was validated by the Pratt-Phillips Laboratory at North Carolina State University by dilutional parallelism between kit standards and recovery. All samples were diluted 10% based on kit instructions, run in duplicate and repeated on subsequent runs if CV >15%.

*5.3.5. Statistical Analysis*

Statistical analysis was performed in GraphPad Prism Version 10.2.3 (GraphPad Software, Boston, MA) and SAS Studio Version 3.82 (SAS Institute, Inc., Cary, NC). Descriptive statistics (mean, standard deviation) were calculated, with values presented as mean ± standard deviation (SD). In GraphPad Prism, analysis of variance (ANOVA) for repeated measures was performed on the different variables (e.g., ferritin, hepcidin) across the treatment groups (denoted as IRON, CTRL) and time points (denoted as 0, 3, 6, 12 and 24 hours) of the IAT during each study phase (denoted as Pre and Post). Additionally, multiple comparisons were utilized. Finally, area under the curve (AUC) was determined on variables (e.g., hepcidin) and figures were prepared.

In SAS Studio, the PROC MIXED procedure for repeated measures was used to assess the effects of time (0, 3, 6, 12 and 24 hours), treatment (IRON, CTRL) and phase (Pre, Post) on all variables measured (e.g., ferritin, hepcidin). The log of a variable was taken if distribution was deemed abnormal. LS MEANS was used to compute the effect of each variable and a post-hoc Tukey's HSD test was performed. Significance was determined by a P-value of $\leq 0.05$ while a trend was noted if $0.05 < P \leq 0.1$.

**5.4. Results**

For the iron dose across the two IATs, horses received a range of 52,000-82,000 mg of ferrous sulfate. As ferrous sulfate is only 20% elemental iron, horses received a range of 10,400-

16,400 mg of elemental iron, which is well over the daily iron requirement for horses based on the NRC (NRC, 2007).

Ferritin values during the Pre IAT are presented as mean ± standard deviation (SD) across the 2 treatment groups and 5 time periods in Table 5.4. There was a trend for a time effect (P=0.1), however, interestingly, ferritin values fluctuated throughout the test, with these decreasing across both groups from 0 to 3 hours. Additionally, ferritin values at 0 hours were above the reference range (43-261 ng/ml) provided by the diagnostic laboratory. However, at the end of the IAT, ferritin values were below baseline values.

Serum iron values during the Pre IAT are presented in Table 5.5. There was a significant time effect (P<0.0001), in which serum iron increased from the 0- to 3-hour time point. When using multiple comparisons, there was a significant time effect between 0- and 3-hours for both IRON and CTRL horses (IRON: P=0.007, CTRL: P=0.01). Additionally, 3-hour serum iron values were above provided reference ranges for both IRON and CTRL horses. However, serum iron values did return to baseline levels in both groups of horses after 24 hours (Table 5.5).

Total iron binding capacity (TIBC) during the Pre IAT are presented in Table 5.6. Similarly, to serum iron, there was a significant time effect (P<0.0001), in which TIBC increased in both CTRL and IRON horses from 0 to 3 hours. When utilizing multiple comparisons, there was a significant increase of TIBC in IRON horses between 0 and 3 hours (P<0.0001) and between 3 and 24 hours (P=0.003) while a trend was noted in CTRL horses for differences in TIBC between 0 and 3 hours (P=0.07).

Serum hepcidin values during the Pre IAT are shown in Table 5.7. There was a significant time effect (P<0.0001) across the IAT while there was a trend noted for a time x treatment group interaction (P=0.1). When utilizing multiple comparisons, there was a significant

increase in hepcidin values for both IRON (P=0.02) and CTRL (P=0.03) horses between 0 and 12 hours (Table 5.7).

After the Supplement Phase, ferritin values for the Post IAT are shown in Table 5.8. There was a significant time effect (P=0.05) but no treatment effect (P=0.2) was found. When utilizing multiple comparisons, ferritin values were significantly different between IRON and CTRL horses at 3 hours (P=0.008) (Table 5.8). Additionally, ferritin levels in IRON horses were above reference ranges (43-261 ng/ml), especially at the 0-hour time point. Area under the curve (AUC) was calculated for ferritin values between IRON and CTRL horses, in which an unpaired t-test test did not find a significant difference between the two groups (P=0.6) (Figure 5.2). When comparing ferritin values in the Pre and Post IAT in IRON horses, this was significant (P=0.01) (Figure 5.3). Finally, a paired-t test found 0-hour ferritin values during the Pre and Post phase IATs in IRON horses to be significantly different (P=0.02).

Serum iron values during the Post IAT are shown in Table 5.9. There was a significant time effect (P=0.01), in which serum iron values increased in IRON horses between 0 and 3 hours. Serum iron values were also above the provided reference range (73-140 ug/dl), especially in IRON horses throughout the IAT. None of the comparisons between the 5 different time points were significant in CTRL horses when using multiple comparisons, however, in IRON horses there was differences seen between 3 and 24 hours (P=0.004), 6 and 12 hours (P=0.01) and 12 and 24 hours (P=0.004) (Table 5.9). When comparing values of serum iron between the Pre and Post phase IAT in iron-supplemented horses, there was a trend noted for differences between the two phases (P=0.06) (Figure 5.4). Values for total iron binding capacity during the Post phase IAT are shown in Table 5.10. There was a time (P=0.05) but no treatment (P=0.7)

effect. Interestingly, TIBC values generally returned to baseline levels by the end of the IAT (Table 5.10).

Serum hepcidin values across the 5 time points during the Post IAT are shown in Table 5.11. There was a significant time (P=0.01) effect as well as a trend noted for a time x treatment group interaction (P=0.1). Multiple comparisons revealed differences in hepcidin in IRON horses between 0 and 12 hours (P=0.0004), 0 and 24 hours (P=0.01) and 3 and 12 hours (P=0.03) while none of the time comparisons were significant in CTRL horses (Table 5.11). Area under the curve (AUC) was calculated for hepcidin values in IRON and CTRL horses. An unpaired t-test revealed that IRON horses had a higher hepcidin response (P=0.01) compared to CTRL horses (Figure 5.5). Additionally, when AUC was calculated for serum hepcidin between the Pre and Post phase IATs in IRON horses, a paired t-test revealed a trend (P=0.1) between these two phases.

When using PROC MIXED to assess the effects of time, phase and treatment during the iron absorption tests, there was a phase x treatment interaction (P=0.002) for ferritin (Table 5.12), in which ferritin concentrations did increase in IRON horses between the two phases (Figure 5.3). There was a significant difference between the two phases (P<0.05) for serum iron as well as a trend noted between the two treatments (P=0.1) (Table 5.12), in which serum iron concentrations were generally higher in IRON horses compared to CTRL horses. For hepcidin, there was a trend (P=0.1) noted for differences between the Pre and Post phase (Table 5.12).

**5.5. Discussion**

To the authors' knowledge, an oral iron absorption test (IAT) has never been documented in horses, in which horses received a large bolus of iron in order to see how iron metabolism parameters change over a 24-hour period. Based on receiving a dose of 25 mg/kg of BW of

elemental iron in the form of ferrous sulfate, it does appear that this dose increases parameters like serum iron and hepcidin over a 24-hour period, and that some of these responses are higher when horses are supplemented with iron over a 28-day period.

Surprisingly, serum ferritin values decreased between 0 and 3-hours during both IATs in both control and iron-supplemented horses. During the Post phase IAT, baseline ferritin levels were higher in IRON horses at 522.5±189.48 ng/ml versus CTRL horses at 414.3±70.49 ng/ml, however after 3-hours these values decreased to 416.5±100.51 and 289.0±14.53 ng/ml in IRON and CTRL horses, respectively. By the end of the IAT, ferritin levels returned to close to baseline, with these being 464.8±130.18 and 389.3±75.14 ng/ml in IRON and CTRL horses, respectively. When comparing ferritin values in iron-supplemented horses between the Pre and Post IATs, the Post IAT saw higher ferritin values throughout the test (P=0.01), indicating that horses supplemented with iron are storing more iron since serum ferritin is generally regarded as the best indicator of body iron status (Smith et al., 1984).

In humans, serum ferritin has been measured when performing iron absorption tests. A small-dose (20 mg) iron test was used to evaluate its potential to measure iron absorption and found that there was an inverse correlation between serum ferritin and maximum serum iron after a 3-hour test (Joosten et al., 1997). In another study evaluating patients with inflammatory bowel disease (IBD), ferritin and pro-hepcidin were evaluated during an iron absorption test. In IBD patients, there were no associations between peak serum iron with ferritin and pro-hepcidin, however, ferritin was associated with peak iron in controls. Thus, ferritin can predict iron absorption but this may not always be plausible when inflammatory cytokines may be present, especially in instances like IBD (Hamid-Jan et al., 2008).

Serum iron is a measure of iron within the blood while total iron binding capacity (TIBC) measures the blood's ability to bind iron with transferrin, which is an important transporter that can hold up to 2 iron atoms and transports iron within the bloodstream (Abbaspour et al., 2014; Dev & Babitt, 2017). Both serum iron and TIBC peaked at 3 hours after the oral iron dose during both IATs, in which serum iron values in IRON horses during the Post phase IAT were 237.2±150.74 ug/dl at 0 hours and 323.4±79.65 ug/dl at 3 hours. For TIBC, these values in IRON horses were 373.7±63.08 ug/dl at 0 hours and 401.4±56 ug/dl at 3 hours. Similar to serum ferritin, there was a trend noted for differences between serum iron during the Pre and Post phase IAT in iron-supplemented horses, indicating that serum iron is increasing during an IAT in supplemented horses. In humans, iron absorption tests have shown to increase serum iron, one study finding that 60 mg of iron increased serum iron significantly within 2 hours in iron-deficiency anemia patients (Rondinelli et al., 2017) and similar results were seen in healthy subjects administered 100 mg of iron (Hoppe et al., 2004).

The authors were surprised about serum ferritin decreasing 3 hours after a large dose of iron. However, when visually comparing serum iron and ferritin concentrations in iron-supplemented horses during the Pre and Post phase IAT, it does appear that serum iron peaks while serum ferritin levels are lowest at 3 hours during the absorption test (Figure 5.6). This is in line with humans studies, in which an inverse correlation was found between peak serum iron and serum ferritin after an absorption test (Joosten et al., 1997). A potential reason why serum iron is highest when serum ferritin is lowest during an absorption test could be due to a higher amount of iron being circulated within the blood (resulting in higher serum iron) instead of being utilized for iron storage (resulting in lower serum ferritin), although this relationship needs to be explored further in horses.

Hepcidin, a peptide hormone produced by the liver, is thought to be the master regulator of systemic iron homeostasis (Camaschella et al., 2020; Dev & Babitt, 2017), in which it acts by controlling iron within the circulation. For example, in times of high iron, such as with increased serum iron and iron overload, hepcidin expression will be increased and bind to ferroportin, an iron transporter, to block iron from being released into the bloodstream (Andrews & Schmidt, 2007), thus resulting in less iron being circulated. During both IATs, hepcidin values generally peaked around 12 hours in IRON horses, with these values being 80.1±23.87 and 79.1±21.43 ng/ml for the Pre and Post phase IAT, respectively. Additionally, during the Post phase IAT, multiple comparisons found a significant increase between 0- and 12-hour hepcidin levels in IRON horses, with these values increasing from 44.7±18.08 to 79.1±21.43 ng/ml over the 12-hour span. This increase could indicate that hepcidin expression is being upregulated in order to block iron from the circulation due to increased serum iron and iron within the body, although this relationship could be explored further. While there is little information about serum hepcidin levels in horses, a recent study noted levels of 110±40 ng/ml (Wijekoon et al., 2022), which is similar to the present study. Finally, when calculating AUC, hepcidin responses were higher in iron-supplemented horses compared to control horses during the final IAT.

Oral IATs and hepcidin levels have been looked at in humans in order to diagnose iron issues such as iron-deficiency anemia. Using an IAT, a recent study found that patients with a low serum hepcidin level had anemia associated with gastrointestinal tract abnormalities (Loveikytea et al., 2023). Additionally, patients with high hepcidin levels were genetically tested and confirmed to have iron-refractory iron-deficiency anemia, which causes mutations in a gene that can cause uncontrolled hepcidin expression (Donker et al., 2016; Loveikytea et al., 2023). Another study assessed plasma hepcidin levels in women who were either iron-deficient or iron-

sufficient and underwent an iron test (Stoffel et al., 2020; Zimmermann et al., 2009). Plasma hepcidin and ferritin were strongly correlated and plasma hepcidin predicted iron bioavailability from sources of iron (e.g., ferrous sulfate and fumarate), thus indicating hepcidin could predict bioavailability of iron (Zimmermann et al., 2009). Although there is limited data, especially in horses, performing an iron absorption test and measuring serum hepcidin could be utilized as a diagnostic tool to assess iron bioavailability as well as the functionality of hepcidin at controlling iron circulation.

Despite the present study yielding promising results for the potential use of an IAT as a diagnostic tool, one main limitation is the iron dose utilized. As there is no prior research published in which horses underwent this type of test, the present dose was based on a previous study where ponies did not exhibit adverse effects when fed excess iron (Pearson & Andreasen, 2001). Therefore, future studies in the form of titration studies should be done to assess the effects of varying levels of an iron dose given and how the response of iron metabolism parameters could change. Additionally, the type of iron given could affect the responses, therefore other sources of iron could be tested to see if this affects results. Regardless, the present study does show validity in using an iron absorption test to assess iron metabolism parameters such as ferritin, serum iron and hepcidin.

### 5.6. Conclusion

The results of the present study indicate that iron supplementation influences serum ferritin, serum iron and hepcidin in horses undergoing an iron absorption test. Ferritin was higher in iron-supplemented horses when compared to controls, with ferritin levels being above the provided reference range, indicating iron-supplemented horses are likely storing more iron. Serum iron was also higher in iron-supplemented horses, and while serum iron levels peaked at

3-hours after an iron dose, serum ferritin levels were lower at this time point. Additionally, hepcidin responses were higher in iron-supplemented horses when compared to control horses, indicating a higher expression of this peptide hormone in order to block iron from being circulated within the bloodstream. Although further studies need to assess different amounts of an iron dose as well as iron source, this pilot study shows that an oral iron absorption test could be used as a diagnostic tool to assess iron status within the body and potential health issues related to iron metabolism in horses.

## Tables and Figures

**Table 5.1.** Complete nutrient analysis of orchardgrass-timothy hay used during the first 28 days of the study (Hay Phase) completed by the North Carolina Department of Agriculture and Consumer Services

| Analyte | Content[a] |
|---|---|
| Dry Matter (%) | 89.80 |
| Crude Protein (%) | 8.48 |
| Digestible Energy (mcal/lb) | 0.91 |
| Calcium (%) | 0.37 |
| Phosphorus (%) | 0.17 |
| Sodium (%) | 0.02 |
| Copper (ppm) | 4.90 |
| Iron (ppm) | 53.63 |
| Zinc (ppm) | 12.34 |
| Ash (%) | 6.72 |

[a]Nutrient values are presented on a 100% dry matter basis.

**Table 5.2.** Guaranteed analysis of SmartVite Thrive Pellets (Smart Pak Equine, Plymouth, MA) per 1 scoop (40 g) using during the study

| Analyte | Content |
|---|---|
| Crude Protein (%) | 5% |
| Calcium (%) | 1.75 (min), 2.25 (max) |
| Phosphorus (%) | 1.1 |
| Salt (%) | 2.5 (min), 2.6 (max) |
| Magnesium (mg) | 1500 |
| Potassium (mg) | 450 |
| Copper (ppm) | 312.5 |
| Zinc (ppm) | 937.5 |
| Vitamin A (IU) | 7500 |
| Vitamin D3 (IU) | 750 |
| Vitamin E (IU) | 125 |

Ingredients: Dehydrated Alfalfa Meal, Corn Distillers Dried Grains with Solubles, Magnesium Oxide, Monocalcium Phosphate, Calcium Carbonate, Salt, Lignin Sulfonate, Monosodium Phosphate, Heat Stabilized Rice Bran, Vegetable Oil, Flaxseed Meal, Selenium Yeast, Dried Kelp, Vitamin E Supplement, Calcium Propionate (preservative), Zinc Polysaccharide Complex, Zinc Sulfate, Manganese Polysaccharide Complex, Manganese Sulfate, Ascorbic Acid, Copper Polysaccharide Complex, Copper Sulfate, Biotin, Zinc Proteinate, Manganese Proteinate, Copper Proteinate, Thiamine Mononitrate, Niacin, Riboflavin, Vitamin A Acetate, Natural & Artificial Flavors, Menadione Sodium Bisulfite Complex (source of Vitamin K activity), Pyridoxine Hydrochloride, d-Calcium Pantothenate, Folic Acid, Beta Carotene, Vitamin B 12 Supplement,

Ethylenediamine Dihydriodide, Vitamin D3 Supplement, Coconut Oil, Cobalt Carbonate, Sodium Propionate (preservative), Citric Acid (preservative).

**Table 5.3.** Complete nutrient analysis of orchardgrass-timothy hay used during the latter 28 days of the study (Supplement Phase) completed by the North Carolina Department of Agriculture and Consumer Services

| **Analyte** | **Content[a]** |
|---|---|
| Dry Matter (%) | 90.42 |
| Crude Protein (%) | 8.83 |
| Digestible Energy (mcal/lb) | 1.01 |
| Calcium (%) | 0.34 |
| Phosphorus (%) | 0.08 |
| Sodium (%) | - |
| Copper (ppm) | 4.27 |
| Iron (ppm) | 74.11 |
| Zinc (ppm) | 14.99 |
| Ash (%) | 5.20 |

[a]Nutrient values are presented on a 100% dry matter basis.

**Figure 5.1.** Oral iron dose administered to study horses during iron absorption tests (IAT)

**Table 5.4.** Serum ferritin values (measured in ng/ml) during the Pre phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There were no significant time or treatment effects

| | **Ferritin (ng/ml)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 318.7±90.93 | 436.0±194.35 |
| **3** | 267.5±75.48 | 376.7±205.02 |
| **6** | 252.5±80.39 | 413.3±254.6 |
| **12** | 288.8±112.87 | 375.3±170.42 |
| **24** | 278.7±82.56 | 379.3±209.84 |

[a]Time is denoted in hours

**Table 5.5.** Serum iron values (measured in ug/dl) during the Pre phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time ($P<0.0001$) effect and significant differences utilizing multiple comparisons between time periods are indicated by different letters within table columns

| | **Serum Iron (ug/dl)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 150.7±21.98[a] | 143.9±33.09[g] |
| **3** | 274.0±54.31[b,c] | 237.4±51.95[h,i] |
| **6** | 236.7±42.89[d] | 186.7±52.05[j] |
| **12** | 188.3±30.01[e] | 140.9±48.13 |
| **24** | 153.8±41.62[f] | 136.8±28.90 |

[a]Time is denoted in hours

**Table 5.6.** Serum TIBC values (measured in ug/dl) during the Pre phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P<0.0001) effect and significant differences utilizing multiple comparisons between time periods are indicated by different letters within table columns

| | **Total Iron Binding Capacity (TIBC) (ug/dl)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 395.8±28.82[a] | 411.1±25.93 |
| **3** | 449.9±25.28[b,c] | 460.9±38.29[g] |
| **6** | 411.3±34.94[d,e] | 399.7±40.97[h] |
| **12** | 394.3±38.31 | 405.9±30.26 |
| **24** | 379.1±45.1[f] | 391.0±41.30 |

[a]Time is denoted in hours

**Table 5.7.** Serum hepcidin values (measured in ng/ml) during the Pre phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P<0.0001) effect and significant differences utilizing multiple comparisons between time periods are indicated by different letters within table columns

| | **Hepcidin (ng/ml)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 54.4±18.42[a] | 62.2±9.92[c] |
| **3** | 62.3±19.10 | 68.1±13.69 |
| **6** | 78.9±29.90 | 96.6±29.74 |
| **12** | 80.1±23.87[b] | 82.1±17.23 |
| **24** | 55.9±19.65 | 48.6±10.39[d] |

[a]Time is denoted in hours

**Table 5.8.** Serum ferritin values (measured in ng/ml) during the Post phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P=0.05) effect and significant differences utilizing multiple comparisons are indicated by different letters within table rows

| | **Ferritin (ng/ml)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 522.5±189.48 | 414.3±70.49 |
| **3** | 416.5±100.51[a] | 289.0±14.53[b] |
| **6** | 419.9±146.94 | 297.0±15.72 |
| **12** | 463.0±147.89 | 383.0±45.57 |
| **24** | 464.8±130.18 | 389.3±75.14 |

[a]Time is denoted in hours

**Figure 5.2.** Area under the curve for serum ferritin values (measured in ng/ml) between control (denoted in blue) and iron (denoted in red) horses during the Post phase IAT. An unpaired t-test revealed no significant differences (P=0.6) in ferritin responses between control and iron horses

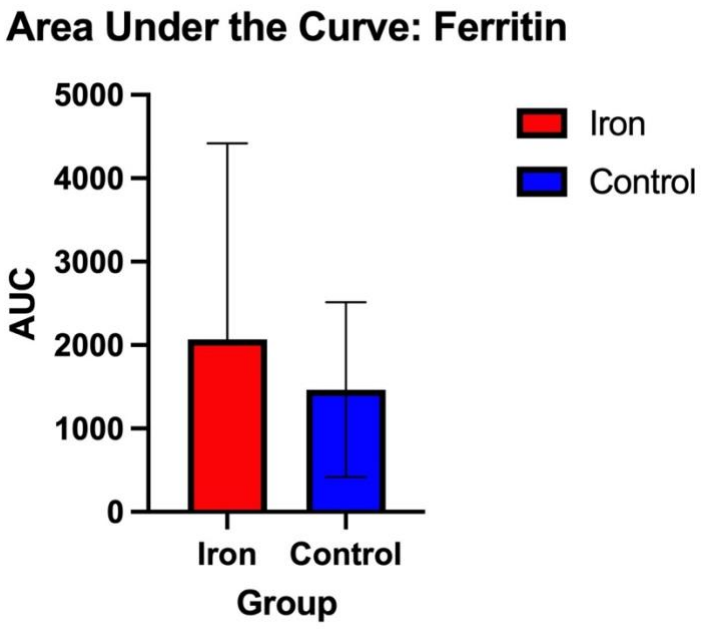

**Figure 5.3.** Serum ferritin (measured in ng/ml) values during the Pre (denoted in solid pink line) and Post (denoted in solid purple line) IATs in iron-supplemented horses only at 0, 3, 6, 12 and 24 hours. There was a significant (P=0.01) phase effect between the two IATs

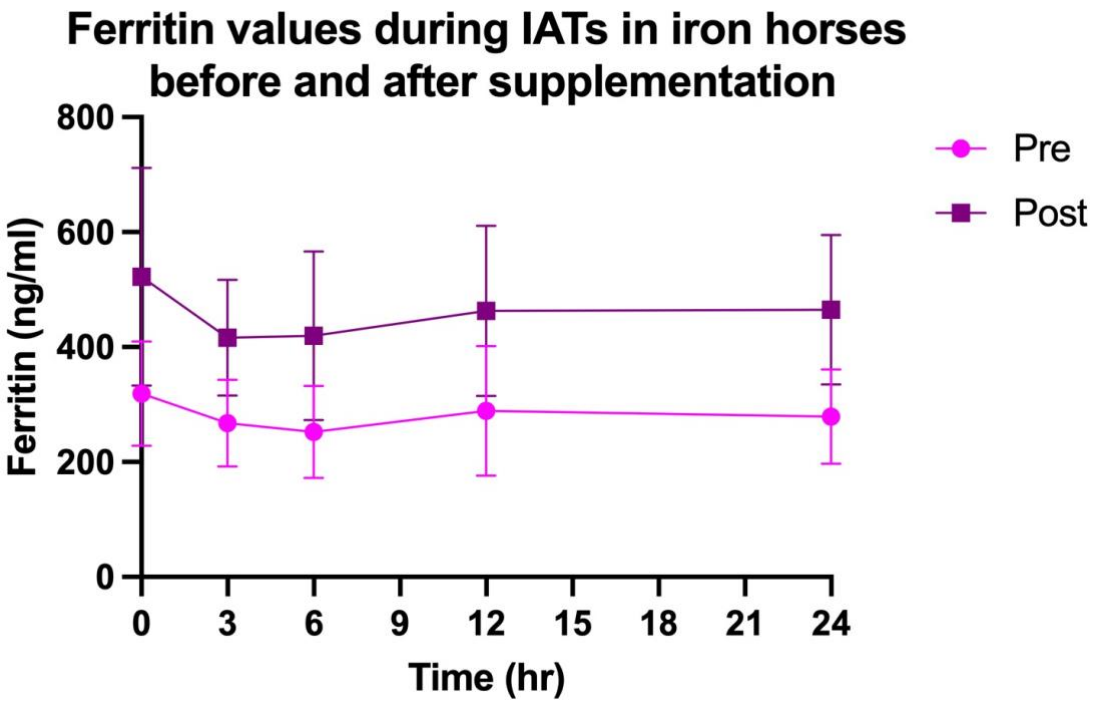


**Table 5.9.** Serum iron values (measured in ug/dl) during the Post phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P=0.01) effect and significant differences utilizing multiple comparisons between time periods are indicated by different letters within table columns

| | **Serum Iron (ug/dl)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 237.2±150.74 | 179.4±26.14 |
| **3** | 323.4±79.65[a] | 300.0±39.86 |
| **6** | 304.6±69.52[c] | 286.2±49.45 |
| **12** | 250.1±66.52[d] | 241.0±60.06 |
| **24** | 180.7±78.93[b,e] | 216.7±41.79 |

[a]Time is denoted in hours.

**Figure 5.4.** Serum iron (measured in ug/dl) values during the Pre (denoted in solid pink line) and Post (denoted in solid purple line) IATs in iron-supplemented horses only at 0, 3, 6, 12 and 24 hours. There was a trend for a phase (P=0.06) effect between the two IATs

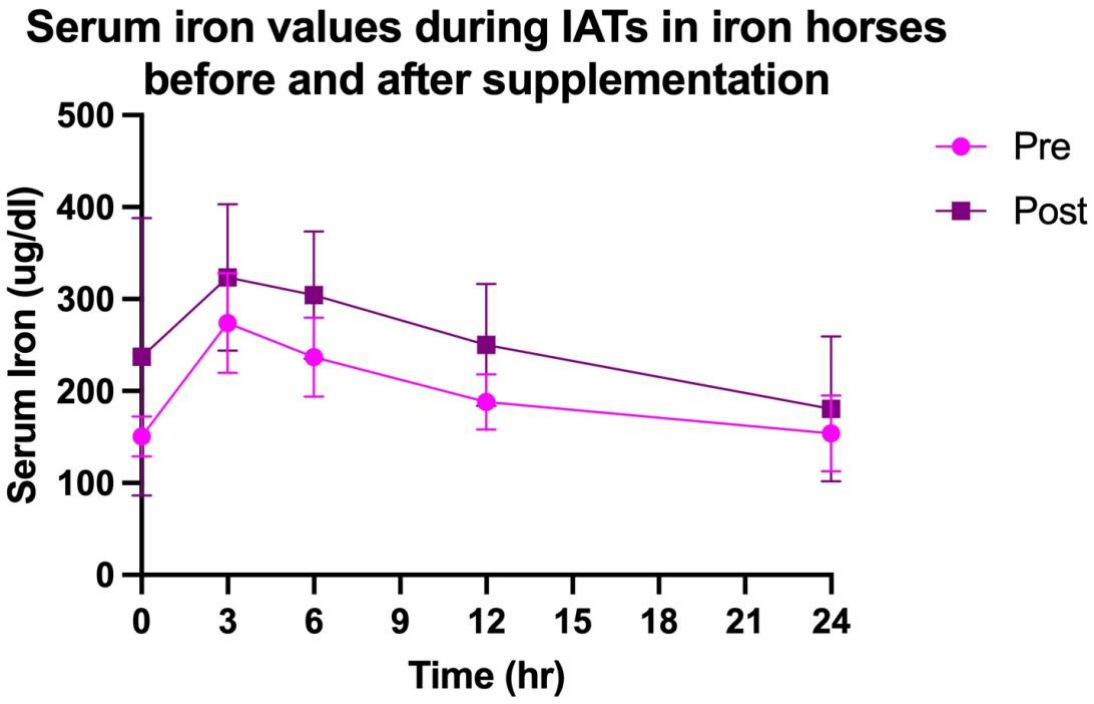


**Table 5.10.** Serum TIBC values (measured in ug/dl) during the Post phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P=0.05) effect

| | **Total Iron Binding Capacity (TIBC) (ug/dl)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 373.7±63.08 | 357.7±5.26 |
| **3** | 401.4±56.00 | 387.9±14.38 |
| **6** | 390.6±59.84 | 369.0±20.82 |
| **12** | 369.9±44.20 | 365.1±31.81 |
| **24** | 348.5±37.68 | 346.1±14.35 |

[a]Time is denoted in hours

**Table 5.11.** Serum hepcidin values (measured in ng/ml) during the Post phase IAT in iron and control horses at 0, 3, 6, 12 and 24 hours. All values are presented as mean ± standard deviation (SD). There was a significant time (P=0.01) effect and significant differences utilizing multiple comparisons between time periods are indicated by different letters within table columns

| | **Hepcidin (ng/ml)** | |
|---|---|---|
| **Time[a]** | **Iron** | **Control** |
| **0** | 44.7±18.08[a,c,e] | 51.4±16.17 |
| **3** | 52.5±20.11[g] | 62.5±11.62 |
| **6** | 73.4±23.98[b] | 62.3±25.43 |
| **12** | 79.1±21.43[d,h] | 61.7±27.58 |
| **24** | 69.0±25.80[f] | 58.6±20.55 |

[a]Time is denoted in hours

**Figure 5.5.** Area under the curve for serum hepcidin values (measured in ng/ml) between control (denoted in blue) and iron (denoted in red) horses during the Post phase IAT. An unpaired t-test revealed significant differences (P=0.0158) in hepcidin responses between control and iron horses

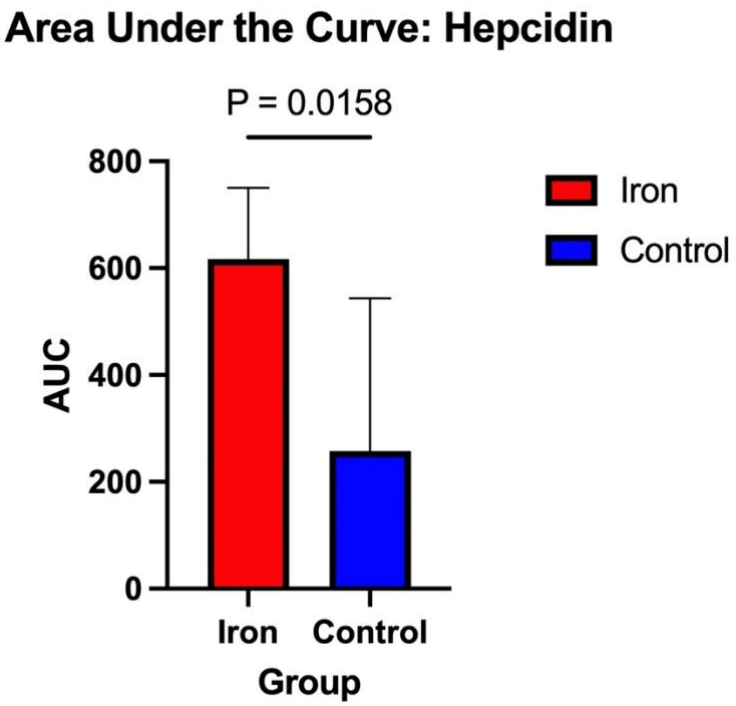

**Figure 5.6.** Serum iron (measured in ug/dl) and ferritin (measured in ng/ml) values during the Pre (serum iron denoted in dashed green line, serum ferritin denoted in solid pink line) and Post (serum iron denoted in dashed blue line, serum ferritin denoted in solid purple line) IATs in iron-supplemented horses only at 0, 3, 6, 12 and 24 hours

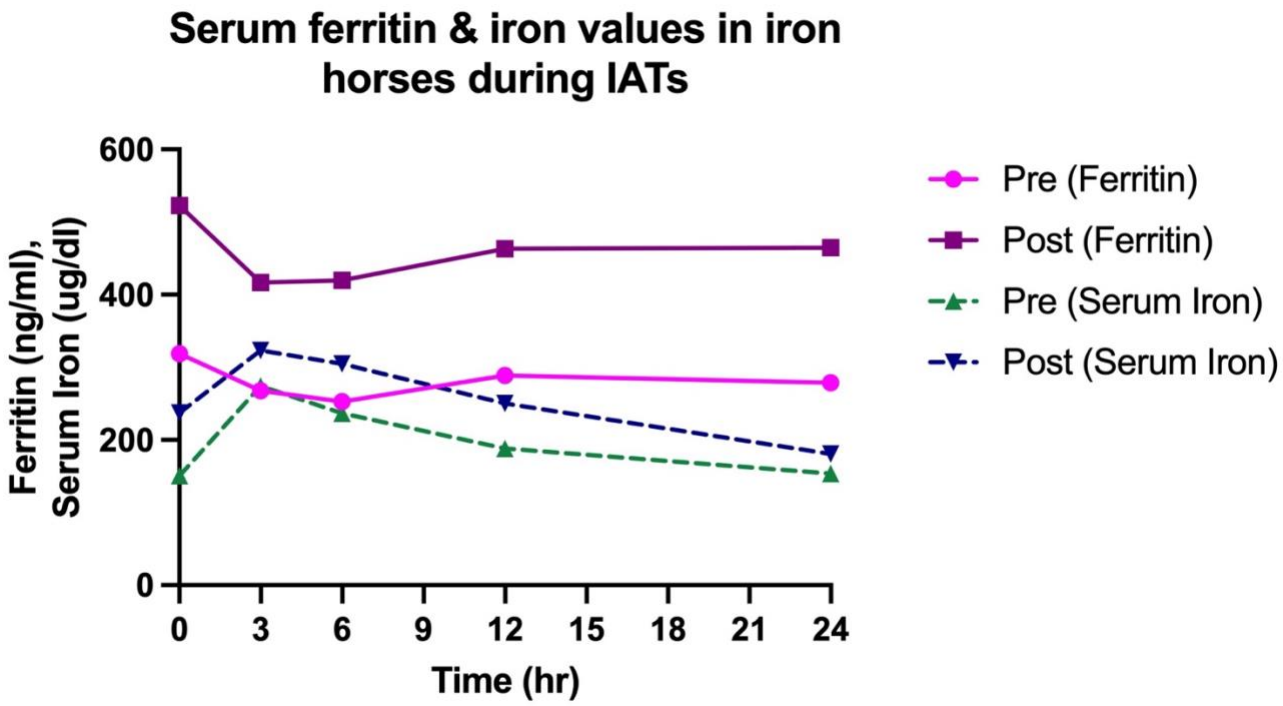

**Table 5.12.** Effect of phase (Pre, Post IAT), treatment (iron, control) and time (0, 3, 6, 12, 24 hours) on ferritin (measured as ng/ml), serum iron (measured as ug/dl), TIBC (measured as ug/dl) and hepcidin (expressed as ng/ml) during an iron absorption test

| | **Fixed Effects, *P-value*[4]** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Variables** | **Phase[1]** | **Treatment[2]** | **Time[3]** | **Phase* Treatment** | **Phase* Time** | **Treatment* Time** | **Phase* Treatment*Time** |
| **Ferritin (ng/ml)** | 0.01 | NS | NS | 0.002 | NS | NS | NS |
| **Serum Iron (ug/dl)** | 0.0001 | 0.1 | <0.0001 | NS | NS | NS | NS |
| **TIBC (ug/dl)** | 0.01 | NS | 0.04 | NS | NS | NS | NS |
| **Hepcidin(ng/ml)** | 0.1 | NS | 0.001 | NS | NS | NS | NS |

[1]Phase consisted of: Pre, Post IAT
[2]Treatment consisted of: Iron, control
[3]Time consisted of: 0, 3, 6, 12 and 24 hours
[4]Statistical significance set at $P \leq 0.05$ while a trend was noted if $0.05 < P \leq 0.1$

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# CHAPTER 6: STUDY 5

## The relationship of TNF-alpha, insulin and body condition in elite competition ponies

### 6.1. Abstract

Prior research has shown relationships between inflammation, obesity and insulin dysregulation in ponies and/or horses. Additionally, TNF-alpha, a pro-inflammatory cytokine, has been shown to be elevated in ponies affected with equine metabolic syndrome. Thus, the aim of this study was to measure TNF-alpha concentrations in samples from elite competition ponies and see if there is a relationship between these, basal insulin levels and body condition. All samples were taken from 23 ponies at the 2021 USEF Pony Finals (Lexington, KY), where two researchers assigned body condition scores (BCS) and cresty neck scores (CNS) based on the Henneke and Carter system, respectively. Jugular venous samples were taken and glucose was measured from whole blood samples then serum was aliquoted and frozen until analysis. Basal serum insulin was analyzed during a previous study via a radioimmunoassay kit and measured in pmol/L. TNF-alpha values were analyzed with a commercially available ELISA kit and measured in pg/ml. Correlation analysis was performed in GraphPad Prism Version 10.2.3 (GraphPad Software, Boston, MA) to assess the relationships between BCS, CNS, insulin, TNF-alpha and glucose. Out of the 23 ponies assessed, 91.3% of ponies were considered fleshy or obese while 60.9% had a cresty neck. TNF-alpha and insulin values were 5803 ± 9249 pg/ml and 25.85 ± 43.67 pmol/L, respectively, and there was a trend for a small, positive relationship (Pearson r = 0.37; P=0.07) between these. Additionally, there was a strong, moderate relationship (Pearson r = 0.61; P=0.002) between BCS and CNS. Although there are a number of factors that could have affected the wide range of TNF-alpha concentrations seen such as pony age and the impact of exercise, it does appear there are some correlations between insulin and

TNF-alpha concentrations, although this could be explored further in a more controlled study. Additionally, the majority of the ponies from the present study were over conditioned, showing a need for education surrounding the dangers of obesity, especially in elite competition ponies.

## 6.2. Introduction

Obesity and inflammation are closely correlated as obesity is considered a chronic mild inflammatory state (Dandona et al., 2004). There are a number of different inflammatory cytokines, however, TNF-alpha, a pro-inflammatory cytokine, is produced by immune cells (e.g., macrophages) and circulating inflammatory cytokines have been found to be increased within the bloodstream of obese humans and horses (Olszanecka-Glinianowicz et al., 2004; Parameswaran & Patial, 2010; Vick et al., 2007). Within the adipose tissue, TNF-alpha can play an influential role, such as inhibiting gene expression which regulates fatty acid and glucose metabolism as well as reducing secretion of adipokines (Henneke et al., 1983). Additionally, TNF-alpha levels have been found to be elevated in insulin-resistant and obese rodent models (Hotamisligil et al., 1993; Sethi & Hotamisligil, 2021; Vick et al., 2007). Thus, it appears that obesity, inflammation, and insulin resistance are closely related.

The incidence of obesity is prevalent within equine populations, in which upwards of 50% of the equine population is considered overweight (Jaqueth et al., 2018; Johnson et al., 2009; Pratt-Phillips et al., 2023; Pratt-Phillips et al., 2010). Ponies are more prone to equine metabolic syndrome (EMS), which is similar to diabetes in humans (Basinska et al., 2015; Fulop et al., 2006). EMS can be caused by a number of risk factors such as obesity, regional adiposity, inflammation and insulin dysregulation, meaning there is a disturbance in glucose metabolism, resulting in altered glucose and insulin levels (Durham et al., 2019; Frank et al., 2010). Ultimately, EMS is a serious issue that could result in euthanasia and is influenced by over

expression of pro-inflammatory cytokines like TNF-alpha and IL-6 (Frank, 2011; Nieto-Vazquez et al., 2008). Ponies with EMS had higher levels of serum TNF-alpha when compared to controls (Basinska et al., 2015). Additionally, TNF-alpha and insulin sensitivity were inversely related in older mares (Vick et al., 2007). Based on the previous literature (Basinska et al., 2015; Frank, 2011; Vick et al., 2007), assessing pro-inflammatory cytokines and insulin levels may be beneficial to better understand the elucidation of obesity-related issues like EMS in horses and/or ponies.

Despite the plethora of issues associated with obesity, this issue is still prevalent within competition horses and ponies (Munjizun, 2023; Pagan et al., 2009; Pratt-Phillips et al., 2023). Pagan et al. (2009) found that insulin and body condition scores (BCS) were higher in pony hunters when compared to other disciplines of show horses while Pratt-Phillips et al. (2023) found that ponies with a higher BCS had higher model scores, indicating that judges are rewarding excessive adiposity in show ponies (Pagan et al., 2009; Pratt-Phillips et al., 2023). Additionally, in a survey-based study, judges were more lenient towards overweight horses (Munjizun & Phillips, 2021). Therefore, education surrounding the dangers of obesity and potential issues associated with it needs to be emphasized in order to ensure the health and performance of horses and ponies is kept in mind, especially at the elite competition level.

There is a need to continue to elucidate the relationship between pro-inflammatory cytokine (e.g., TNF-alpha) expression, obesity and insulin levels in horses to better understand how these influence each other. With the dangers of obesity and EMS being more prevalent in ponies, the present study aimed to measure TNF-alpha concentrations and see if there is a correlation between these concentrations, basal insulin levels and body condition in a population of elite competition ponies.

## 6.3. Materials and Methods

The North Carolina State University Institutional Animal Care and Use committee approved this study. Serum samples and previous data utilized for the present study were taken during a prior study (Munjizun, 2023) and the protocol is outlined below.

### *6.3.1. Animals*

This study was conducted at the 2021 USEF Pony Finals in Lexington, Kentucky, held August 10-15, 2021. Participants were recruited via emails sent to the competitors and via onsite solicitation for volunteers. Owners/representatives signed a waiver allowing the researchers to use their ponies. In total, 58 ponies were included in the prior study (Munjizun, 2023).

Ponies were assessed the day after a pony's final competition, which consisted of a jumping class. Two experienced researchers assessed the ponies' body condition scores (BCS) and cresty neck scores (CNS) using the systems developed by Henneke et al. and Carter et al. respectively, both by visual and palpation (Carter et al., 2009; Henneke et al., 1983). All ponies evaluated during the study were considered healthy.

### *6.3.2. Blood Collection, Glucose and Insulin Measurement*

Owners and representatives were asked to not feed their ponies grain prior to assessment. Evacuated tubes containing no anticoagulant were used to collect blood samples from the pony's jugular vein. Blood collection was performed following the assessment of BCS and CNS. Blood glucose measurement was performed using a hand-held glucometer (One-Touch Ultra 2, LifeScan, Inc., Milpitas, CA). The use of a hand-held glucometer on horses was validated previously (Hackett & McCue, 2010). Following the glucose measurement, blood was allowed to clot. Centrifugation was performed at 1200 x *g* for 15 minutes, and then serum was collected into

microcentrifuge tubes (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA). Serum samples were stored at -4°C for later use.

Measurement of insulin concentrations was performed on the serum samples. Insulin concentrations were measured using a commercial radioimmunoassay kit (Kit #HI-14K, EMD Millipore Corporation, Billerica, MA), a Human Insulin Kit that has been validated for equine insulin analysis (Spears et al., 2020). Samples were analyzed in duplicates with intra-assay CV 1.6%.

*6.3.3. TNF-alpha Analysis*

Measurement of TNF-alpha concentrations was performed on serum samples. TNF-alpha concentrations were measured on a commercial ELISA kit (Equine TNF-alpha DuoSet ELISA, R&D Systems, Inc., Minneapolis, MN) and has been validated previously (Lavoie-Lamoureux et al., 2010). The kit was validated by the Pratt-Phillips laboratory at North Carolina State University by dilutional parallelism between kit standards. Samples were analyzed in duplicates and diluted as necessary. Concentrations are presented as pg/ml.

*6.3.4. Statistical Analysis*

All statistical analysis was performed in GraphPad Prism Version 10.2.3 (GraphPad Software, Boston, MA). Descriptive statistics (mean, standard deviation, ranges) were calculated, with values presented as mean ± standard deviation (SD). Correlation analysis using Pearson's correlation was performed on the variables body condition score, cresty neck score, glucose, insulin and TNF-alpha with the association between variables determined based on the correlation coefficient and P-value, with a significance level of $P \leq 0.05$ while a trend was noted if $0.05 < P \leq 0.1$. Additional Pearson's correlation was performed on ponies considered obese (BCS >7) and ponies with a cresty neck (CNS ≥ 3).

## 6.4. Results

A total of twenty-three serum samples were analyzed for TNF-alpha values from the fifty-eight initially collected during the prior study (Munjizun, 2023). Table 6.1 displays the means ± standard deviation (SD) and ranges of body condition scores (BCS), cresty neck scores (CNS), glucose (mmol/l), insulin (pmol/l) and TNF-alpha (pg/ml) across the 23 samples.

Just under nine percent (8.7%; 2/23) of the ponies sampled were considered moderate ($4<BCS<6$) while 65.2% (15/23) were considered fleshy ($6\leq BCS\leq 7$). Finally, 26.1% (6/23) of ponies were considered obese (BCS > 7). For cresty neck scores (CNS), 39.1% (9/23) of ponies had an ideal neck (CNS < 3) while 60.9% (14/23) of ponies had a cresty neck (CNS ≥ 3).

Basal insulin concentrations ranged from 2.94 – 212.7 pmol/L (equivalent to 0.4 – 30.63 μU/ml) in the study population (Table 6.1). Just over four percent (4.3%; 1/23) of ponies sampled from the present study would be diagnosed with insulin dysregulation (ID) when using a cut-off value of 138.9-347.2 pmol/L (equivalent to 20-50 μU/ml) for insulin, which is indicative of ID when forage quality is unknown (Durham et al., 2019; Frank et al., 2022), with an insulin value of 212.7 pmol/ (equivalent to 30.63 μU/ml). TNF-alpha concentrations were 5803 ± 9249 pg/ml across all ponies (Table 6.1) while fleshy and obese ponies had a mean value of 6286 ± 9555 pg/ml.

There was a trend for a small, positive relationship between insulin and TNF-alpha concentrations (Pearson r = 0.37; P=0.07) when considering all ponies. Additionally, there was a strong, moderate relationship between BCS and CNS (Pearson r = 0.61; P=0.002) as well as between insulin and glucose concentrations (Pearson r = 0.7; $P<0.05$) (Figure 6.1). When considering only ponies that were obese (BCS > 7), insulin and glucose concentrations were found to be strongly correlated with each other (Pearson r = 0.85; P=0.02). Finally, for ponies

with a cresty neck (CNS ≥ 3), there was a trend for a small, moderate relationship (Pearson r = 0.4; P=0.1) between insulin and TNF-alpha concentrations.

### 6.5. Discussion

The majority of the ponies included in the present study were over conditioned, with 91.3% of the total twenty-three ponies considered fleshy or obese and 60.9% of the ponies having a cresty neck. Additionally, there was a moderate, positive relationship between body condition score and cresty neck score as well as a trend noted for a relationship between insulin and TNF-alpha concentrations. Only one pony was considered hyperinsulinemic, however the prior study in which the present study utilized samples from found that 45.5% of obese (BCS > 7) ponies and 55.6% of ponies with a cresty neck (CNS ≥ 3) were hyperinsulinemic (Munjizun, 2023).

Obesity and over conditioning are common within equine populations (Jaqueth et al., 2018; Pratt-Phillips et al., 2023; Pratt-Phillips et al., 2010). Obesity can lead to a plethora of issues such as insulin resistance, laminitis and others, ultimately compromising performance and health (Johnson et al., 2009). Despite horses being used for performance, previous studies have also found that riding or competition horses are over conditioned (Harker et al., 2011; Wyse et al., 2008), with Pagan et al. (2009) finding that pony hunters had higher body condition scores and plasma insulin compared to horses in other disciplines (Pagan et al., 2009). Additionally, Pratt-Phillips et al. (2023) found that there was a positive relationship between body condition score and model score, meaning over conditioned ponies received higher scores from the judges (Pratt-Phillips et al., 2023). Thus, it appears that obese ponies are commonly seen and excessive adiposity is being rewarded by judges, which is dangerous for the health of ponies.

There was a wide range of TNF-alpha concentrations (14.83 – 31,420 pg/ml) seen in the twenty-three samples assessed. However, several samples not included in the analysis were deemed undetectable, thus showing a large range in these concentrations within the present population of ponies sampled. In a study by Lavoie-Lamoureux et al. (2010), TNF-alpha values ranged from undetectable to 500,000 pg/ml and these were higher in horses with heaves, a common airway inflammatory disease, when compared to controls (Lavoie-Lamoureux et al., 2010). In another study assessing TNF-alpha concentrations in ponies with EMS and healthy ponies, ponies affected with EMS had higher levels of serum TNF-alpha with a mean of 1.970 μg/ml (equivalent to 1,970,000 pg/ml) compared to healthy ponies (Basinska et al., 2015). The significantly higher TNF-alpha values seen in Basinka et al., (2015) versus the present study could be due to a different assay utilized (Basinska et al., 2015). TNF-alpha has also been looked at in older, obese horses, with Adams et al. (2009) finding that reducing body weight and fat in older horses decreased serum TNF-alpha protein levels (Adams et al., 2009). In the present study, there was a trend of a relationship between insulin and TNF-alpha concentrations in all ponies. This relationship has also been explored in previous studies, in which Vick et al. (2007) found that TNF-alpha was inversely related to insulin sensitivity in older mares (Vick et al., 2007).

TNF-alpha is a pro-inflammatory cytokine produced by immune cells such as macrophages and monocytes (Parameswaran & Patial, 2010) and has been seen within the bloodstream of obese humans (Olszanecka-Glinianowicz et al., 2004) as well as horses (Vick et al., 2007). TNF-alpha has been shown to inhibit regulation of fatty acid and glucose metabolism in the adipose tissue. Additionally, increased TNF-alpha affects the activity of an insulin receptor (e.g., tyrosine kinase) and is correlated with laminitis, obesity and insulin resistance (Henneke et

al., 1983; Hotamisligil & Spiegelman, 1994; Treiber et al., 2009). Ageing is often associated with a chronic inflammatory state dubbed “inflamm-aging” and increased TNF-alpha has been seen in older humans (Brüünsgaard & Pedersen, 2003; Franceschi et al., 2000) as well as horses (Adams et al., 2009).

Despite seeing correlations between insulin and TNF-alpha as well as body condition score and cresty neck score in the present study, there were some limitations to the study. First, the sample size of the present study was small compared to the previous study in which samples was obtained from, where serum from 58 ponies was analyzed for insulin (Munjizun, 2023). Therefore, if more samples were able to be analyzed for TNF-alpha concentrations, results may have been more indicative of a relationship between insulin, TNF-alpha and body condition. Second, the present study could not determine a potential relationship between age and TNF-alpha concentrations. As age seems to have an influence on inflammatory cytokines (Adams et al., 2009; Brüünsgaard & Pedersen, 2003; Franceschi et al., 2000), future studies could assess the potential relationships between body condition, insulin, TNF-alpha, and age. Finally, blood samples were obtained from the ponies 1-2 days after the competition was complete, in which the last day of competition for the study population was jumping. Exercise has been shown to induce changes in TNF-alpha (Liburt et al., 2010), thus some of the higher levels of TNF-alpha seen in the present study could be attributed to this. Therefore, future studies should attempt to control confounding factors in order to more accurately assess TNF-alpha concentrations, especially in competition horses and/or ponies.

### 6.6. Conclusion

Ponies assessed during the present study were typically over conditioned and had a cresty neck. Despite only one pony being considered hyperinsulinemic, there was a trend seen for a

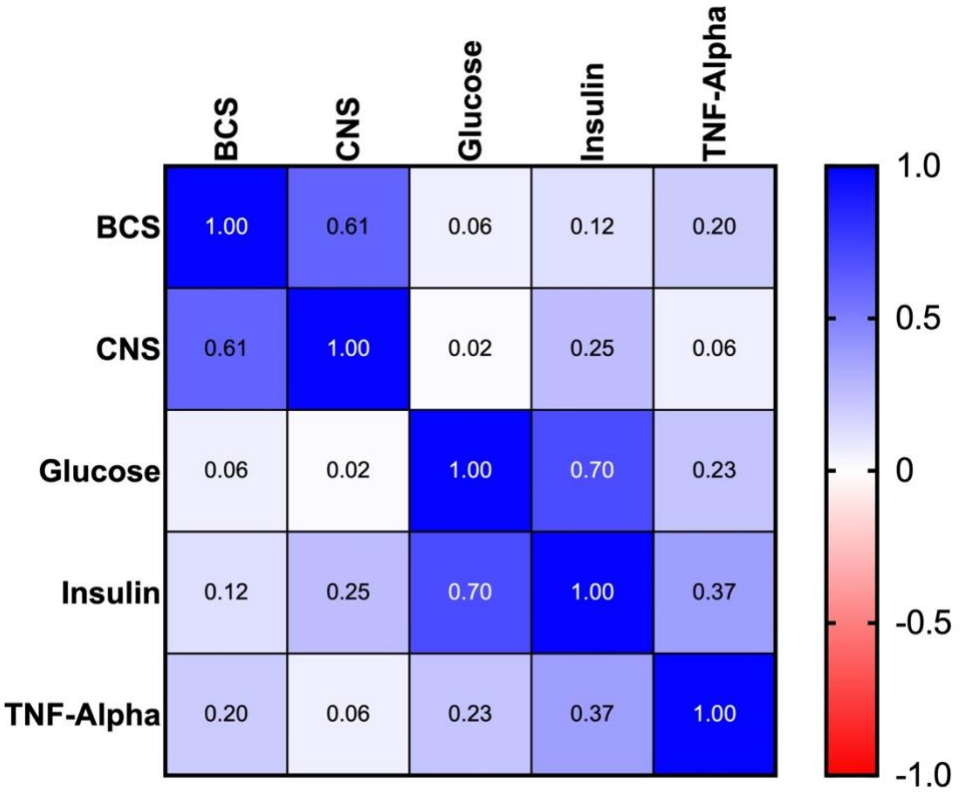
relationship between insulin and TNF-alpha concentrations. As TNF-alpha could be influenced by a number of different factors such as obesity, age, exercise and others, future studies assessing this pro-inflammatory cytokine should try to control confounding factors to further determine if there is a relationship between insulin, TNF-alpha, age and body condition in competition ponies and/or horses. Overall, education on the dangers of obesity needs to be emphasized, especially in competition horses and ponies.

## Tables and Figures

**Table 6.1.** Means, standard deviations and ranges of variables measured in elite competition ponies (n=23) at the 2021 USEF Pony Finals

| Variable | Mean | Range |
|---|---|---|
| Body condition score | 6.67 ± 0.64 | 5.25 – 8 |
| Cresty neck score | 2.95 ± 0.68 | 1.5 – 4 |
| Glucose (mmol/L) | 92.78 ± 12.2 | 77 – 134 |
| Insulin (pmol/L) | 25.85 ± 43.67 | 2.94 – 212.7 |
| TNF-alpha (pg/ml) | 5803 ± 9249 | 14.83 – 31,420 |

**Figure 6.1.** Correlation matrix showing Pearson correlation coefficients (r) between different variables assessed in elite competition ponies (n=23) at the 2021 USEF Pony Finals. Cells are colored according to whether the correlations are positive (blue) or negative (red) and shaded according to the strength of the relationship based on the Pearson correlation coefficient. Statistical significance was set at P≤0.05 while a trend was noted if 0.05<P≤0.1



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# CHAPTER 7: STUDY 6

## Performance analysis of show jumping rounds at a national pony competition

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### 7.1. Abstract

Performance analysis is utilised by coaches and athletes to identify areas to work on in training and to identify strengths in athlete performance in various sports. However, performance analysis is not commonly used within equestrian sports. The purpose of this study was to evaluate minors and their ponies competing in show jumping at a national pony competition to see if course variables affected performance. All jumping rounds were watched online. Type of faults (e.g., rails, refusals, time faults, fall of horse and or rider), type of fence (e.g., vertical, oxer), approach angle, section of the course where fault(s) occurred and round time were recorded. Spearman's Correlation assessed if round time was correlated to total faults and a series of Kruskal-Wallis analyses determined if significant differences in faults occurred between sections of the course, where these existed, post hoc tests established where differences occurred between rounds. There was no significant difference in total faults across the 4 rounds of competition and no meaningful correlation between round time and total faults (r=0.34; P=0.008). There were no differences between fence type and faults although more faults occurred at verticals (51.7%, n=46 faults at verticals versus 48.3%, n=43 at oxers; P=0.610). Faults were more likely to occur during the final quarter of the course (32.6%, n=29) when

compared to the first quarter (23.6%, n=21; P=0.028). These results showed that faults were more likely to occur in the final quarter of a round. The information gained from this performance analysis could be beneficial to inform training or riding strategies, especially when preparing for a competition.

### 7.2. Introduction

Performance analysis is a technique that evaluates identified factors in order to provide objective feedback and enhance performance within a sport. With this feedback, athletes can improve decision-making in order to increase success in future competitions (Marlin and Williams, 2020; Nicholls *et al.*, 2019). This technique needs to occur within a specific context and is used concurrently with an athlete, their coach(s) and a performance analyst to use information gained from this analysis to help with informing skill development, designing training plans and competition strategies aligned to key performance indicators (McGarry, 2009). Once a particular goal is defined and set, the analyst will describe, explain and predict an athlete's performance by identifying associations between actions (sport-specific behaviours) and outcomes (goals or key performance indicators) while considering external factors such as other competitors and the environment. Through this process, performance improvement strategies can be developed (Marlin and Williams, 2020; McGarry, 2009; Williams, 2013).

Even though performance analysis has been implemented within sports such as football and rugby, it hasn't been commonly used within the equestrian sport of show jumping. The objective of show jumping is for a horse and rider combination to complete a course consisting of jumping obstacles within a specific time or in the fastest time without accruing any penalties, also known as faults. Jumping obstacles can vary based on the competition level but fence heights typically range from 0.70 to 1.60 meters. Fence width must exceed the height by 5 to 15

cm for oxers (a fence with 2-3 rails or poles that can be set at the same or different heights) ((USEF), 2022b).

Due to the welfare of both horse and rider being an increasingly important priority (Campbell, 2021), there has been an increased need to use evidence-based practices across different equestrian sports (Mills *et al.*, 2005). Performance analysis techniques could provide approaches and strategies for riders to utilise evidence-based practices when making decisions about training and preparing for competitions while keeping the welfare of the horse in mind (Marlin and Williams, 2020; Waran and Randle, 2017). Equine performance analysis has mainly focused on subjective assessments through 'feel' or observation of a performance (Ely *et al.*, 2010; Williams, 2013). However, using these subjective assessments relies on memory recall and are dependent on individual perception of a performance, thus being prone to bias. Analysing equestrian performance is complex as it requires focus on the performance of the horse (which can be influenced by the rider), performance of the rider (influenced by the horse), the partnership between the horse and the rider and the 'performance' as a whole entity (Williams, 2015). This complex partnership between horse and rider is exacerbated further since most equestrian athletes train individually (often in isolation) so self-analysis is common compared to human sports. However, the complexity of the horse and rider partnership parallels dynamics seen within human team sports, where performance analysis has been successful (Francis and Jones, 2014; Groom and Cushion, 2004). Despite the complexities seen within equestrian sports, performance analysis techniques can be successfully applied to collect objective data to enable riders, trainers and coaches to make informed decisions when implementing training and competition strategies in order to enhance performance (Marlin and Williams, 2020).

While there have been some studies done within the equestrian sport of show jumping using performance analysis (Marlin and Williams, 2020; Williams *et al.*, 2022) to analyse horse and rider performance, these studies have focused on professional athletes competing in Fédération Equestre International (FEI) competitions. To the authors' knowledge, there are no studies currently published looking at performance analysis within minor riders competing in show jumping. In human sports, utilising video analysis in amateur ice hockey has been shown to be useful to both coaches and youth athletes to help with athletic development and progression to the next level within the sport (Lee, 2011). This could also be applied to minors competing in equestrian sports to help with short-term performance improvement of a horse and rider combination as well as long-term development for the rider to progress to elite levels of show jumping. Therefore, implementing performance analysis to provide evidence-based strategies to improve training and preparing for competitions should be beneficial to minor riders competing in show jumping.

The objective of this study was to evaluate minors and their ponies (under the height of 147 cm) competing in show jumping to see if the course variables studied affected performance using notational analysis, a technique designed to evaluate competition strategies (Duthie *et al.*, 2003). This technique was used during this study to characterise faults, defined as the knocking down of any obstacle or standard with any portion of the rider or horse when jumping the obstacle, a disobedience (refusal) at a fence or fall of horse and or rider ((USEF), 2022b). It was hypothesised that faults were more likely to occur at verticals versus oxers and during the final section (divided into quarters) of the course versus the others.

### 7.3. Materials and Methods

Riders and ponies evaluated for this study competed at the 2021 United States Equestrian Federation Pony Finals (USEF) in Lexington, Kentucky, USA in the Pony Jumper Championship. All riders that competed were considered amateur riders and under the age of 18. Consent of riders to be evaluated for this study according to the North Carolina State University Institutional Review Board was not needed due to rider age and other information being available publicly online. Ponies were all required to be under the height of 147 cm according to competition rules established by USEF ((USEF), 2022a). Riders and their ponies qualified for this competition by accumulating points throughout the year in order to be eligible to compete.

The competition occurred from August 12-14, 2021 at the Kentucky Horse Park in Lexington, Kentucky. All rounds of the competition were held outdoors in a German Geo Textile (GGT) footing arena. The competition consisted of 4 rounds across 3 days of competition. Each round of competition had a different course; however, the number of jumping efforts varied between 12 to 14. Fence heights throughout the rounds of competition were between 1.05 to 1.15 m. All rounds of the competition were recorded by videographers at the competition and then streamed live on the USEF Network[1]. The live recordings were also available to be streamed after the competition so recordings of the rounds were watched online following the conclusion of the competition.

Notational analysis is a methodology that provides insight into the technical demands of various sport activities such as the equestrian sport of show jumping. This is accomplished by using video recording and quantifying movement patterns that indicate skilled performance in relation to an athlete's performance goals (Duthie *et al.*, 2003). This method was applied to evaluate performance at this particular competition to see if there was a relationship between

[1]Footnote: Competition recordings from the USEF Pony Finals Pony Jumper Championship can be accessed at https://www.usef.org/network.

identified course variables studied and faults. Fences were classified by jumping effort, jump type (oxer or vertical), approach angle (left approach [more than 45° from previous fence], right approach [more than 45° from previous fence] or straight approach [4 or more strides following from a previous fence or a turn]) and correct canter lead on approach to the fence (yes or no). These classifications were adapted from a previous study (Marlin and Williams, 2020). Fault type (refusal, rail or fall of horse and or rider), section of the course (divided into quarters based on the number of jumping efforts), round time, time faults and total number of faults were recorded. If a rider and pony combination produced a fault-free round or were excused (eliminated) was also assessed.

*7.3.1. Data Analysis*

Frequency analysis identified patterns in fault accumulation and jumping effort number, jump type, approach angle to the fence and the location of the fence on the course. Spearman's Correlation examined if relationships existed between fault accrual and jumping effort number sequentially across the jumping rounds reviewed. Kruskal Wallis analysis determined if differences occurred between faults accrued and the distribution of faults across the quarters of the course; subsequent post-hoc Mann Whitney U analyses identified where differences in fault accrual existed between each quarter of the course. Significance was set at $P<0.05$.

**7.4. Results**

A total of 23 rider and pony combinations were seen during the first round of competitions. Once the first round of competition was complete, the number of combinations did change slightly as some did not qualify to compete in subsequent rounds. However, the number of combinations competing ranged from 14 to 23 throughout the competition. The ages of riders ranged from 11 to 17 years of age and ponies were all under the height of 147 cm.

Courses had an average of 13.25 jumping efforts, 6.5 verticals, 6.75 oxers and 2 combinations (denoted as *a* and *b*, however for analysis these were deemed individual fences counted incrementally) (Table 7.1). Oxers made up the majority of fence types (50.94%) versus verticals (49.06%). The time allowed to complete the round(s) ranged from 74 to 80 seconds, the average time allowed was 77 seconds. Throughout 78 total rounds of competition, the majority of combinations were within the time allowed with only 3 rounds accruing time faults.

78 total rounds occurred with 20 (25.5%) being a fault-free round (no accrual of faults and completed within the time allowed) and the remaining 58 (74.4%) had faults produced (knocking a rail, refusal, fall of horse and or rider or over the time allowed). Spearman's Correlation found no meaningful correlation between round time and the total number of faults (r=0.34; P=0.008).

*7.4.1. Fence Type*

When comparing the type of fence and faults throughout the competition, 46 faults occurred at verticals (51.7%) and 43 occurred at oxers (48.3%), however there was no significant difference found between type of fence and fault accrual (P=0.610). The majority of faults (70.6%; n=12) occurred at verticals within combinations while 29.4% (n=5) occurred at oxers but no significant difference in fault accrual was found between fence types (P=0.411).

*7.4.2. Fence Approach Angle*

Fence approach angle was defined as left, straight or right when evaluating performance at this particular competition. Throughout the competition, 18% of faults occurred on left approaches, 13.5% on right approaches and 68.5% on straight approaches. However, no significant differences were found between these different fence approach angles (P=0.576).

*7.4.3. Sections of the Course*

Different sections (divided into quarters) of the course were assessed during this analysis. Kruskal-Wallis Analyses found that there was a significant difference (P=0.014) for fault occurrence between the four different quarters of the course. Post-hoc analysis with a Bonferroni adjustment identified that faults were more likely to occur during the final quarter of the course (32.6%, n=29) when compared to the first quarter (23.6%, n=21; P=0.028) (Figure 7.1).

**7.5. Discussion**

Performance analysis of minors and their ponies competing in show jumping at the 2021 USEF Pony Finals was conducted in order to see if the course variables studied affected performance. To the authors' knowledge, this was the first study conducted using performance analysis to evaluate minor and/or amateur riders competing at this particular competition. Although the results indicated that not all course variables studied were statistically significant, there were evident patterns of how faults accrued. The results also demonstrated that notational analysis is an inexpensive and effective way to help inform training and riding strategies, especially when preparing for competitions.

*7.5.1. Faults and Section of the Course*

It was expected that faults were more likely to occur during the final quarter of the course versus the others. Based on the results, faults were found to be statistically significant during the final quarter of the course versus the 1$^{st}$. This is in agreement with a study by Marlin and Williams (2020), who found that the number of faults increased between the 1$^{st}$ and 4$^{th}$ quarters and knock-downs (rails) were 2.8 times more likely to occur within the second half of the course versus the first.

One possible reason for the increase in fault accrual during the final quarter of the course versus the others could be from pony and rider fatigue, influenced by a number of factors. As this competition was held outdoors and during the summer, environmental factors such as the weather could have played a role in fatigue. The classes for this particular competition were typically held in the hottest part of the day. Based on this, riders and ponies may be more likely to physically fatigue easier. Increased fault accrual could also be due to the rider experiencing mental fatigue towards the end of the course. Since the sport of show jumping involves a partnership amongst horse and rider where the rider is primarily making the decisions, this could be considered both a physically and cognitively-demanding sport. Mental fatigue is defined as a psychobiological state that is caused by extended periods of demanding cognitive activity (Marcora *et al.,* 2009). A systematic review revealed that mental fatigue has a negative impact on skilled performance, including decision-making and technical skills in human sports such as basketball and soccer (Sun *et al.,* 2021). Therefore, mental fatigue could be influencing a rider's concentration and decision-making skills as the amount of time spent in this cognitively-demanding activity continues, resulting in increased fault accrual seen within later sections of the course.

*7.5.2. Fence Type*

Faults at vertical fences were expected to occur more frequently versus at oxers. Traditionally, riders and coaches have considered vertical fences more difficult to successfully jump (Marlin and Williams, 2020). However, there was no significant difference between the type of fence (verticals or oxers) and occurrence of faults, although more faults occurred at verticals (51.6%, n=46) versus at oxers (48.3%, n=43). This is in agreement with Marlin and William's (2020) findings, in which faults were found to be more common at verticals but this

was not significantly associated with increased faults. Another study found that the highest probability of faults occurred at vertical obstacles with water (Ničová and Bartošová, 2022), and although the present study did not have water obstacles, faults did occur more at verticals. The lack of influence of fence type found in the present study and Marlin and William's (2020) study could be due to a horse's jumping kinematics. Walker *et al.* (2018) found that elite show jumping horses use similar techniques to successfully jump over a fence, regardless if the fence is a vertical or oxer. However, since the present study was looking at non-elite ponies and amateur riders, the ponies may not have a consistent jumping technique across the different fence types. Another study found that lower level horses free jumping a 1 meter vertical fence had a significant difference in their back motion versus a group of higher level horses (Cassiat *et al.*, 2004). It is important to note that Cassiat *et al.* (2004) did not assess ponies who would likely free jump at a lower height, thus results could be skewed on jumping technique if ponies were included. Therefore, depending on the level of experience a horse or pony has, jumping technique may play a role on the occurrence of faults at different fence types. Fence design and colour could also be associated with jumping performance. In a recent study, fence design and colour combinations were assessed and it was found that fence design had more of an influence on fault accrual rather than colour in elite show jumping (Williams *et al.*, 2022). Future work from the present study could be undertaken to assess if fence design as well as colour does have an influence on fault occurrence at amateur level competitions.

*7.5.3. Limitations*

One major limitation was the low number of jumping efforts seen throughout the competition. Due to this factor, this could have been the reason why the study failed to see many statistically significant differences even though there were trends seen of how faults accrued.

This study also only evaluated these rider and pony combinations at this particular competition. If these combinations were evaluated longitudinally, this could help identify patterns within particular courses and these specific combinations, thus allowing for a more accurate analysis of performance. Finally, all rounds were evaluated through competition recordings posted on the USEF Network so the performance analyst was reliant on the videographer and could not manipulate the angles at which particular fences were seen so some course variables (such as approach angle) could have been recorded inaccurately based on the angle seen within the video. Despite these limitations, notational analysis used within the present study provides evidence that performance analysis could be beneficial to inform training and riding strategies within the sport of show jumping.

### 7.6. Conclusion

Information gained from performance analysis techniques could be beneficial to inform training or riding strategies, especially when preparing for competitions. As the welfare of horses and riders becomes increasingly important, utilising these techniques could provide evidence-based approaches for riders to use when training and competing while keeping the welfare of the horse in mind. Therefore, research within performance analysis should continue to be done and this technique should be advocated to be used regularly within the equestrian sport of show jumping.

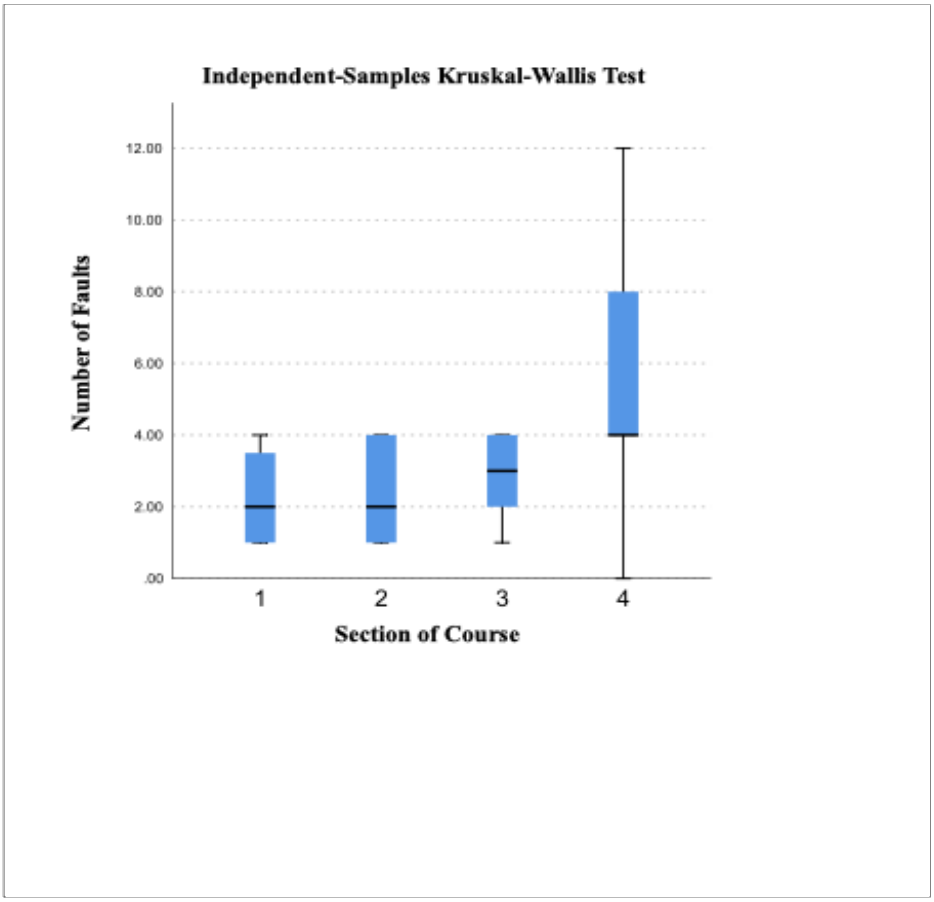
## Tables and Figures

**Table 7.1.** Jumping variables and time allowed across the 4 rounds of competition[A]

| | Round 1 | Round 2 | Round 3 | Round 4 |
|---|---|---|---|---|
| **Jumping efforts** | 13 | 14 | 14 | 12 |
| **Verticals** | 7 | 7 | 6 | 6 |
| **Oxers** | 6 | 7 | 8 | 6 |
| **Combinations** | 2 | 2 | 2 | 2 |
| **Fence height** | 1.05 meters | 1.10 meters | 1.10 meters | 1.05 meters |
| **Time allowed** | 74 seconds | 80 seconds | 76 seconds | 78 seconds |

[A]Footnote: Breakdown of the number of jumping efforts, verticals, oxers, combinations, fence height and time allowed across the four rounds of competition observed for analysis.

**Figure 7.1.** Fault accrual amongst different sections of the course[B]




[B]Footnote: Representation of fault accrual throughout the different sections of the course, divided into quarters. Results indicated that faults were more likely to occur during the final quarter (denoted as 4) of the course when compared to the first quarter (denoted as 1).

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# CHAPTER 8: GENERAL CONCLUSIONS

Horses are hindgut fermenters, in which they possess an enlarged cecum and colon that house billions of microbes. These microbes break down and ferment fibrous materials to produce volatile fatty acids, which ultimately provide energy to a horse. In Study 1, the fecal microbiome and in-vitro fermentation patterns were assessed in three populations of horses: one feral (Shackleford) and two domesticated (NCSU, privately owned) to determine if there were differences in microbial composition and fermentation patterns within these populations. There were clear differences in microbial composition, in which differential abundance testing revealed differences amongst the populations in taxa such as the phyla Firmicutes, Bacteroidetes and Spirochaetes as well as the orders Spirochaetales, Clostridiales, Erysipelotrichales and Bacteroidales. Additionally, beta diversity revealed clear, visual separations among the feral and domesticated populations, indicating distinct differences of the composition of the microbiota. Acetate was the highest volatile fatty acid produced while butyrate was produced more within the feral Shackleford population. The results of this study indicate that the habitual diet likely plays a role in the composition of the microbiome and subsequent fermentation patterns. With this knowledge, nutrition and management practices in domesticated horses should be evaluated in order to minimize hindgut disturbances to the microbiota.

The next three studies focused on iron metabolism within horses. Iron is an important micromineral involved in several physiological functions and is commonly found within the equine diet in pasture/forages, concentrates and supplements. There are some concerns about increased iron intake and its correlation to increased body iron stores (in the form of ferritin), obesity and insulin dysregulation so this was explored throughout these studies. In study 2, blood samples from mixed-breed horses (n=21) kept on cool-season pasture for the previous four

months were taken to assess the relationship between dietary iron and iron and glucose metabolism parameters. The majority (85%) of horses had ferritin values over the provided reference range, with a mean value of 409.2 ± 164.5 ng/ml while 80% of the horses had serum iron values over the provided reference range, with a mean value of 174.8 ± 41.89 ug/dl. Additionally, there was a moderate, positive relationship between hepcidin and dietary iron intake (Pearson r = 0.57; P = 0.01). Finally, although no correlation was found between insulin and ferritin when assessing all horses, Pearson's correlation did find a strong correlation (Pearson r = 0.8; P=0.01) between ferritin and insulin when considering horses with basal insulin values indicative of hyperinsulinemia. Results from this study indicate further investigation into the impact of dietary iron on serum insulin, ferritin and iron.

Study 3 focused on the effects of iron supplementation on glucose and iron metabolism parameters. Horses were housed individually and after an initial 28 days, horses were randomly assigned to be supplemented with iron (IRON) or continue on a control diet (CTRL) to evaluate the effects of iron supplementation on iron and glucose metabolism parameters. Serum ferritin was higher in IRON horses across the 2 phases (P=0.01), in which values were 522.5±189.48 ng/ml at the end of the study, which is over the provided reference range (43-261 ng/ml). When utilizing an oral sugar test at the end of the study, although 60-min insulin values were not indicative of insulin dysregulation (>65 μU/ml) in IRON horses, insulin responses were significantly higher compared to CTRL horses. Based on Study 3, there seems to be a plausible relationship between increased body iron status and glucose and insulin responses. Further investigation into the correlation between dietary iron, increased body iron status and altered glucose metabolism is warranted.

The last study focused on iron metabolism (Study 4) utilized an oral iron absorption test (IAT) to evaluate iron absorption. Two IATs (denoted as Pre, Post) were performed, in which jugular venous samples were taken and then a large dose of iron was given. Subsequent blood samples were taken at 3-, 6-, 12- and 24-hours post-dose to assess the change in iron metabolism parameters. There was a phase (Pre vs. Post) x treatment (CTRL vs. IRON) interaction for serum ferritin, in which ferritin values increased for IRON horses. Area under the curve (AUC) was calculated for serum hepcidin responses and these were higher in iron-supplemented horses when compared to controls. The results of this study indicate that iron supplementation and giving a large dose of iron will affect iron metabolism parameters like hepcidin and ferritin in horses, however, future studies should evaluate the effects of iron metabolism responses utilizing different doses and sources of iron. Implications from this study showcase a potential investigative method in order to assess iron status and absorption within horses while evaluating for any iron-related issues such as anemia or iron overload.

The aim of Study 5 was to evaluate TNF-alpha, a pro-inflammatory cytokine, which has been shown to be elevated in ponies affected with equine metabolic syndrome, and assess if there are correlations between TNF-alpha, insulin and body condition. Body condition scores (BCS) based on the Henneke system and cresty neck scores (CNS) based on the Carter system were assigned and serum samples were taken from elite competition ponies at the 2021 USEF Pony Finals to evaluate insulin and TNF-alpha concentrations. Out of the 23 ponies assessed, 91.3% of ponies were considered fleshy or obese based on their BCS while 60.9% had a cresty neck. TNF-alpha and insulin concentrations were $5803 \pm 9249$ pg/ml and $25.85 \pm 43.67$ pmol/L, respectively, and there was a trend for a small, positive relationship between these when utilizing Pearson's correlation. Although there are many factors that could have affected the wide range of

TNF-alpha concentrations seen (e.g., pony age, impact of exercise), it does appear that there are correlations between insulin, TNF-alpha and body condition, however this needs to be explored further. Therefore, TNF-alpha values could be useful in evaluating inflammatory status and/or metabolic issues, especially in equine populations prone to obesity.

Performance analysis is used by coaches and athletes to identify areas to work on in training and to ultimately enhance performance in various sports. However, performance analysis is not common in equestrian sports. The purpose of Study 6 was to evaluate ponies and minors competing in show jumping at the 20201 Pony Finals to see if certain course variables affected performance. Jumping rounds were watched online and type of faults, type of fence, approach angle, section of the course where fault(s) occurred and round time were recorded. There were no differences in total faults across the 4 rounds of competition and there were no differences between fence type and faults, although more faults occurred at verticals (51.7%) versus oxers (48.3%). Faults were more likely to occur during the final quarter of the course compared to the first quarter (P=0.028), probably due to fatigue. Results of this type of analysis could be beneficial with informing training or riding strategies, especially when prepping for competitions and developing young riders.

# APPENDICES

## Appendix A

**Figure A1.1.** Bar charts depicting the relative abundance of phyla observed in fecal samples of the three equine populations (Shackleford, NCSU and privately owned) assessed with abundance thresholds set at 0% detection and 70% presence

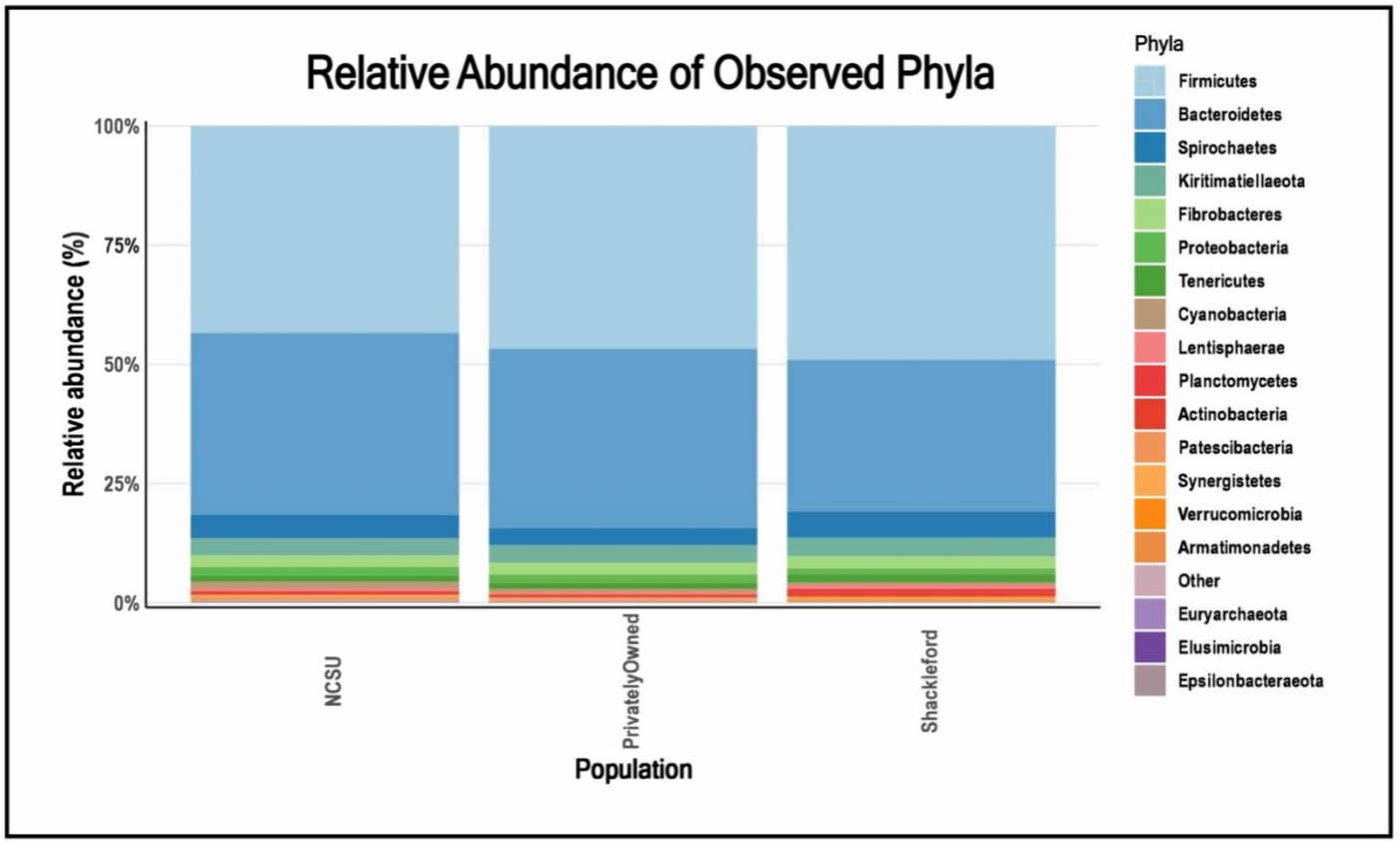
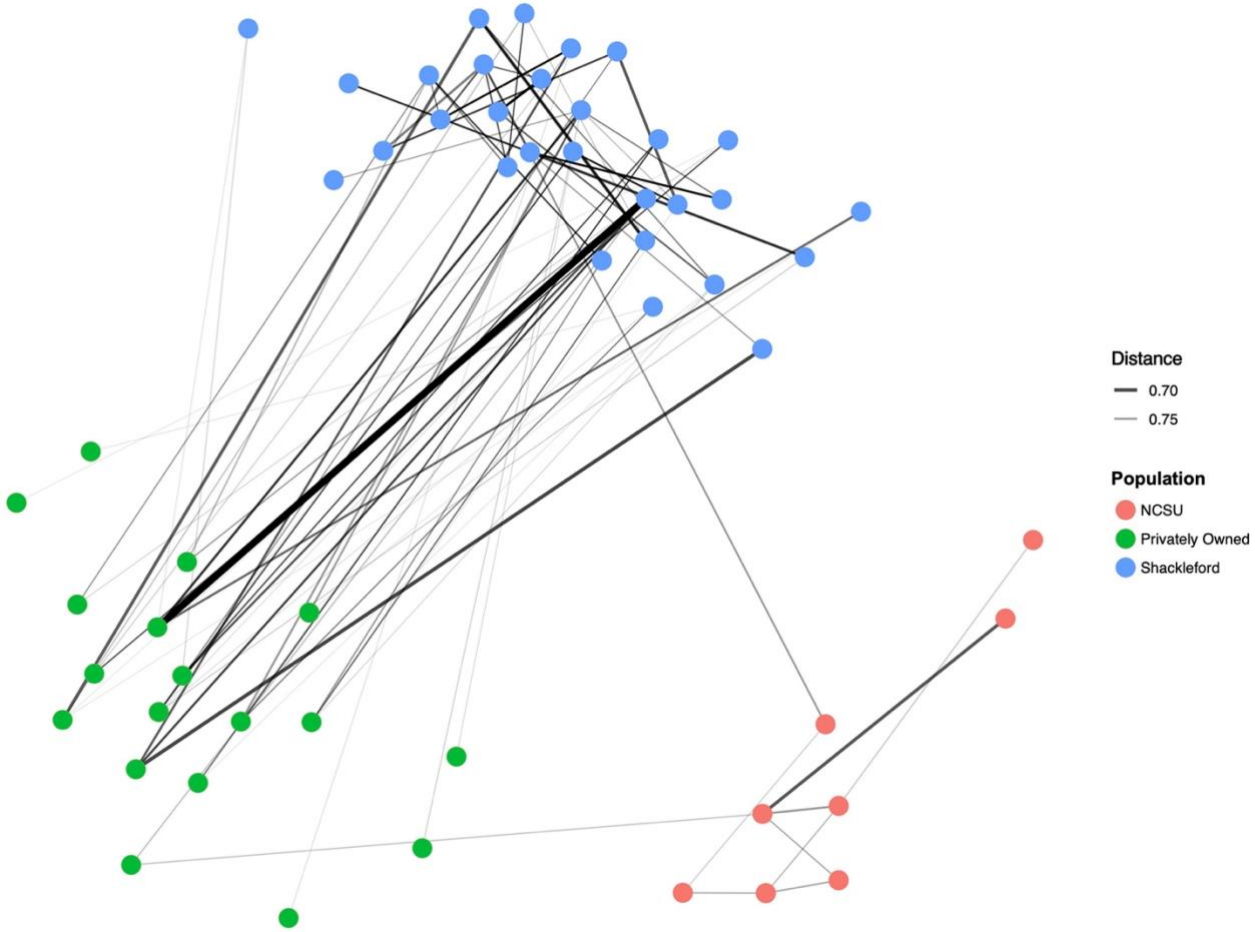


**Figure A1.2.** Microbiome network plot showing distances between amplicon sequence variants (ASVs) utilizing Jaccard Distance. Populations are denoted on the plot as: NCSU (red), privately owned (green) and Shackleford (blue)



# Appendix B

## Validation procedure and results utilizing Horse Hepcidin-Compete ELISA kit (SKU#-HHC-003; Intrinsic LifeSciences LLC, La Jolla, CA) for quantification of hepcidin-25 in equine serum

**Note:** The microwell plates coated with anti-horse hepcidin cannot be split up to be used for multiple runs as the plates will dry out. Plan your experiment accordingly to fill the entire 96-well plate.

## Validation Procedure

1. Prepare all reagents according to kit instructions.
2. Prepare standards and samples according to kit instructions.
    a. Equine serum samples should be run in duplicate at a 10% sample dilution, using 207 µl of Horse Hepcidin Biotin Conjugate and 23 µl of sample to achieve 230 µl/well in the sample set-up plate. Pipette the Horse Hepcidin Biotin Conjugate into the sample set-up plate first before adding the 23 µl of sample. Mix the Horse Hepcidin Biotin Conjugate and sample thoroughly via reverse pipetting in the sample set-up plate before transferring 100 µl/well to the microwell plate coated with anti-horse hepcidin.
    b. Repeat samples if coefficient of variation is greater than 15% on subsequent runs.
3. Stripped horse serum will need to be prepared by stripping serum with charcoal to remove hormones and/or other components. Stripped horse serum should be run on every ELISA plate in duplicate.
4. Dilutional parallelism between kit standards will be performed on 2 separate runs and instructions are outlined below. Dilutional parallelism will not be able to be performed on the same run due to the kit not having sufficient reagents to produce parallel lines at the same time. Stripped horse serum will need to be used. Spike recovery will also be performed on another run and is outlined below.
    a. First dilutional parallelism: Prepare standards and pipette standards into microwell plate before preparing the following dilutions in sample set-up plate.
        i. Dilutional parallelism
            1. P1: Combine Standard (STD) A+B by mixing via reverse pipetting. Pipette 207 µl of Horse Hepcidin Biotin Conjugate and 23 µl of STD A+B into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
            2. P2: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 17.2 µl of STD A+B and 5.7 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
            3. P3: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 11.5 µl of STD A+B and 11.5 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.

4. P4: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 5.7 µl of STD A+B and 17.2 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.

b. Second dilutional parallelism: Prepare standards and pipette standards into microwell plate before preparing the following dilutions in sample set-up plate.
    i. Dilutional parallelism
        1. P1: Combine STD B+C by mixing via reverse pipetting. Pipette 207 µl of Horse Hepcidin Biotin Conjugate and 23 µl of STD B+C into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
        2. P2: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 17.2 µl of STD B+C and 5.7 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
        3. P3: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 11.5 µl of STD B+C and 11.5 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
        4. P4: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 5.7 µl of STD B+C and 17.2 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.

c. Spike recovery: Prepare standards and pipette standards into microwell plate before preparing the following spike recoveries in sample set-up plate.
    i. High recovery: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 11.5 µl of STD A and 11.5 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.
    ii. Low recovery: Pipette 207 µl of Horse Hepcidin Biotin Conjugate, 11.5 µl of STD C and 11.5 µl of stripped horse serum into the sample set-up plate, mix thoroughly via reverse pipetting and pipette 100 µl/well into microwell plate.

## Validation Results

### Data Analysis and Validation Results

*Data Analysis*

Validation of the kit was evaluated for precision (inter-assay and intra-assay coefficients of variation, % CV), dilutional parallelism between kit standards and accuracy (recovery of a known spike). Precision was determined by the following formula:

Coefficient of Variation (% CV) = 100 x (standard deviation/mean hepcidin concentration)

*Validation Results*

An eight-point standard curve from one microwell plate is shown in Figure A2.1.

The average inter-assay and intra-assay coefficient of variation (% CV) for serum hepcidin was 8.7% and 6.7%, respectively.

Dilutional parallelism between kit standards was observed for 1:200, 1:100, 1:50, 1:25 and 1:66, 1:33, 1:16.5 and 1:8.25 in the first and second run, respectively and shown in Figure A2.2.

Accuracy was determined by recovery of a known spike. However, as there are no expected concentrations of equine serum hepcidin at this time, percent recovery was not able to be calculated. Observed concentrations of recoveries completed were: 204.5 and 25.2 ng/ml for the high and low recovery, respectively.

**Figure A2.1.** Eight-point standard curve from one ELISA run

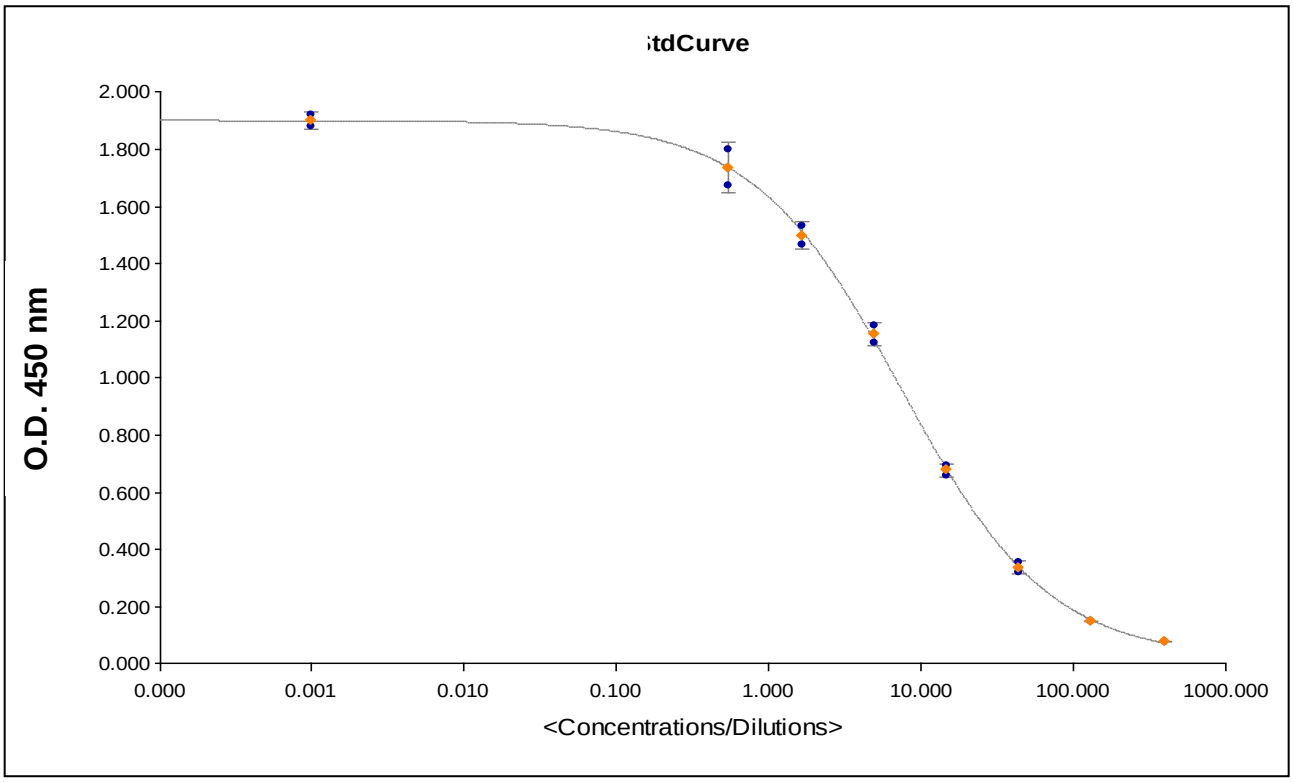



**Figure A2.2.** Dilutional parallelism against the standards