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Evaluation of Equine Fecal pH Measurement Methods and Storage Protocols

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ABSTRACT

The pH of the equine gastrointestinal lumen influences digestion, the microbiome, and enterocyte physiology, each having important impacts on horses. Fecal pH is a simple and noninvasive variable that reflects changes in hindgut pH. The objectives were to evaluate whether there is agreement between fecal pH measured by a meter and pH strips and to determine the effect of storage temperature and time on pH. Fecal samples were collected from 5 broodmares and 8 yearlings on one farm and 12 school horses on another. Feces were collected within 1 min of defecation from an uncontaminated region. A portable pH meter (Hanna Instruments model #9812) and 3 pH strips were used to measure the pH of 4 sub-samples: fresh, 24 h refrigerated at 4° C, 6- and 16-weeks frozen at -20° C. Samples were thawed in a water bath to 20-23° C prior to measurement. Fecal liquid without the addition of distilled water was used for meter readings. The ranges of the 3 pH strips were 4.5-10 (S1), 5.1-7.2 (S2), and 6.0-7.7 (S3). A 1:2 ratio of feces to distilled water was used for pH strip measurements to ensure readability related to color staining. All pH strips had a strong positive correlation (>0.8) with pH meter. Linear regressions between pH measured by strips and meter resulted in r^2 between 0.76 (S1) and 0.86 (S2). Bland-Altman plots quantified agreement between pH meter and S1 (bias 0.005, 95% CI -0.448 to 0.458), S2 (bias -0.033, 95% CI -0.408 to 0.342), and S3 (bias -0.044, 95% CI -0.426 to 0.338). Therefore, S2 best reflects the meter pH, with the highest r^2 and the narrowest Bland-Altman CI. The influence of storage temperature and time were analyzed with a mixed ANOVA. Significance was set at $P<0.05$ and data are presented as means±sem. The pH of fresh and 6-weeks frozen samples was higher than 24 h refrigerated samples (6.49±0.31, 6.47±0.31, 6.39±0.31, $P<0.001$, respectively). No difference was detected comparing the pH of 16-week frozen (6.43±0.31) to other sub-samples. There was a trend for it to be lower than fresh ($P=0.082$). This may indicate relevant change due to long term frozen storage. A difference in the fresh fecal pH ($P<0.001$) was detected using all techniques between the two farms (pH meter results: 6.78±0.11, 6.20±0.09; respectively). The reason for the difference is beyond the scope of this study. The

ability to detect pH differences could be valuable to those interested in management strategies influencing gastrointestinal health. The results of this study indicate that pH strips differ in their ability to reflect pH meter readings, storage temperature and time of storage may influence pH, and pH strips may be useful in detecting relevant differences in fecal pH.

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LIST OF ABBREVIATIONS

C:F	Concentrate to forage ratio
CV	Coefficient of variation NSC Non-structural carbohydrates
DCAD	Dietary cation anion difference
EGGD	Equine Glandular Gastric Disease
EGUS	Equine Gastric Ulcer Syndrome
ESGD	Equine Squamous Gastric Disease
HCl	Hydrochloric acid
LOA	Limits of agreement
pH	Potential of hydrogen
RMSE	Root mean square error
SC	Structural carbohydrates
VFA	Volatile fatty acid

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Chapter 1

Literature Review

1.1 Introduction and Rationale

Horses have a high capacity for athletic ability and require optimum health for maximum performance. Similar to human athletes, high performance horses have a high incidence of gastrointestinal disorders, compromising performance and impacting overall health (Ribeiro et al., 2021). Accurately, inexpensively, and noninvasively evaluating gastrointestinal health is a challenge in the equine industry. Measuring fecal pH is a simple method used as an indirect indicator of hindgut pH and commonly as a response variable in equine nutrition studies (Hydock et al., 2014). The pH of the hindgut may reflect the composition of microbes and digestive efficiency.

To understand the causal relationship between high athletic performance and gastrointestinal disease, differences in diet and exercise of the domesticated horse compared to the wild horse must be noted. Over the past 55 million years, the horse evolved from the dog-sized *Eohippus* into the present-day horse in response to changing environments (Macfadden, 1986). Overall body size, neck, and leg length increased to facilitate continuous grazing on the plains of North America (Janis, 1976). Similarly, the adaptation of the slow eruption of hypsodont teeth throughout a horse's lifetime compensated for the constant grinding of forage. Horses adapted to survive on sparse, low-quality vegetation; their diet predominantly consisted of high fiber and low protein. The hindgut, specifically the cecum and large colon, became the site of fermentation of cellulose and lignin (Janis, 1976).

Wild horses, in their natural setting, spend 16-18 hours grazing and the remainder of the day resting with low levels of daily activity (Goodwin et al., 2002). Faster movements are typically brief and involve fleeing from predators or rivals. The modern 'domesticated' horse, however, is generally subjected to a schedule consisting of two distinct feedings and longer periods of more vigorous physical

activity (Hinchcliff et al., 2014). For example, horses preparing for upper-level competitions may undergo intense training sessions six days a week. To support higher performance requirements, the two traditional feedings consist of more non-structural carbohydrates (NSC), like starch, to fulfill the corresponding higher energy requirements (Davidson and Harris, 2007).

The contrast between the wild horses' diet and exercise routine to those of the domesticated horse is accompanied by novel and exacerbated conditions affecting the gastrointestinal system (Cooper and Albentosa, 2005). The resulting effects on the equine gastrointestinal tract include changes in distribution of blood flow, pH, permeability, and rate of passage. This review serves to assimilate existing data regarding diet and exercise on the pH of different sections of the equine gastrointestinal tract and the impact of pH on gastrointestinal disease. Furthermore, the role of fecal pH as an indicator of gastrointestinal health and protocols used to measure fecal pH will be discussed.

1.2 pH of the Equine Gastrointestinal Tract

1.2.1 Potential of Hydrogen

Potential of hydrogen, denoted as pH, is the measurement of hydrogen ion concentration in a solution. Acids contain more hydrogen ions relative to hydroxide ions; whereas, bases contain more hydroxide ions. Small differences in pH are physiologically significant as pH is measured on a logarithmic scale. For example, a drop in pH from 8.2 to 8.1 indicates a 30% increase in acidity, and a drop from 8.1 to 7.9 indicates a 150% increase in acidity (Bagchi et al., 2013). The pH in the lumen of the gastrointestinal tract is maintained by enterocytes. One mechanism by which enterocytes help maintain a state of homeostasis is through creating a pH gradient to aid digestion and absorption of nutrients (Lallès, 2010). Gastrointestinal pH changes depending on the region and digestive processes occurring (Geor et al., 2013).

1.2.2 Gastrointestinal Anatomy and Physiology

Understanding the parts of the gastrointestinal tract is imperative to understanding the relationship between diet and exercise on gastrointestinal pH. In understanding the "normal" fluctuations in gastrointestinal pH, it can be determined how disease contributes to deviations from the norm.

Horses are non-ruminant herbivores with a digestive tract that can be divided into the foregut and hindgut (Janis, 1976). The foregut is composed of the esophagus, stomach and small intestine. Physical and chemical digestion occurs within the stomach to prepare for enzymatic digestion of starch, proteins, and fats in the small intestine (Rowe et al., 1999). The small intestine is made up of three parts in succession: the duodenum, jejunum, and ileum. The hindgut consists of the cecum, large colon, small colon and rectum. These latter sections are the predominate location of fermentation by a diverse population of microbes (Stevens and Hume, 1998).

Masticated food entering the stomach is predominately subjected to chemical digestion. The stomach makes up 9% of the total tract volume, making it relatively small compared to the remainder of the equine gastrointestinal tract (Geor et al., 2013). The stomach is composed of a lower, glandular region and an upper, non-glandular region. The lower, glandular region contains parietal and chief cells in the mucosa that secrete hydrochloric acid (HCl) and proteolytic enzymes to begin breaking down proteins. Low density digesta/forages float in this upper portion of the stomach (Harris et al., 2010). This food serves to buffer this region from the acids secreted in the glandular region. Forages (pH of 6-7) and saliva (pH of ~7.5) help maintain the higher pH of this region (Ellevik, 2006). Higher density foods such as grain and liquid descend to the lower, glandular region. This region has a pH of 1-2 due to acid secretion (Murray and Grodinsky, 1989).

Digesta continues to travel from the stomach into the duodenum of the small intestine through the pyloric sphincter. Bicarbonate is released into the duodenum to help buffer the low pH of the digesta traveling from the acidic glandular region of the stomach (Geor et al., 2013). This buffering capacity

enables enzymes of the small intestine to function effectively. The small intestine and pancreas secrete enzymes that continue to aid in digestion of proteins, fats, and carbohydrates. Villi in small intestine increase surface area to facilitate absorption of digested nutrients. The pH of digesta traveling through the small intestine progressively becomes more basic: increasing from a pH of ~6.3 in the duodenum to a pH of ~7.1 and ~7.3 in the jejunum and ileum respectively (Mackie and Wilkins, 1988).

The hindgut begins at the intersection termed the ileocecal junction. Digesta travels from the ileum to the cecum and sequentially enters the large colon. The cecum functions as a mixing vat using muscular contractions for slow passage of digesta over a span of approximately 7 hours (Harris et al., 2010). This gives microbes including bacteria, fungi, and protozoa time to begin digesting carbohydrates by fermentation into volatile fatty acids (VFAs) which provide energy for the horse (Geor et al., 2013). Most of the amino acids and proteins produced are largely utilized by microbial bacteria (Stevens and Hume, 1998). Digesta entering the large colon continues to undergo fermentation and passes through in 2-3 days (Harris et al., 2010). There are four sections of the large colon: right ventral, left ventral, right dorsal, left dorsal (Moore et al., 2001). The pelvic flexure joins the ventral and dorsal sections. The colon narrows to slow the passage of digesta for further fermentation. Following the large colon, the last section of the gastrointestinal tract is the small colon, a structure primarily responsible for water absorption. The last 12 inches of the small colon form the rectum where fecal balls exit. Different VFAs are produced throughout the entirety of the hindgut, and the absorption of which can affect gastrointestinal pH (Bergman, 1990). Cecum pH can range from 4.1 to 7.8 and colon pH between 6.3 to 7.5 (Geor et al., 2013). The microbes responsible for fermenting fiber are most efficient at a pH of 6-7 and largely produce the VFAs acetate, propionate, and butyrate (Sjaastad et al., 2003). Absorption of VFAs is essential in maintaining a colon pH of above 6 (van Soest, 1994).

The proportion of different microbes in the hindgut affects the horse's ability to digest and obtain VFAs from certain feedstuffs (Costa et al., 2012). The byproducts generated by different species of microbe influence pH differently. Undigested NSC that reach the hindgut are fermented into lactic acid,

contributing to a more acidic environment (Calsamiglia et al., 2002). Cellulolytic microbes digest structural carbohydrates (SC) into VFAs and ferment more efficiently in a higher pH environment relative to amylolytic microbes. Microbial populations are sensitive to changes in diet composition and in turn pH. Forage-based diets promote gastrointestinal health by contributing to a less acidic environment as a physical buffer protecting the non-glandular region of the stomach and nutrition for cellulolytic microbes in the large intestine (Costa et al., 2012).

Maintaining proper blood and tissue pH is similarly important and implicated in the maintenance of a healthy and diverse microbiome. Blood pH within the normal range of approximately 7.38-7.44 is crucial for optimal enzymatic and cellular function (Bohn, 2013). The respiratory system, kidneys, and secretion of buffering solutions within the gastrointestinal tract are crucial processes for maintaining normal blood pH. Diet can also influence metabolic pH. Adjusting the dietary cation anion difference (DCAD) can help maintain acid-base balance (Hu and Murphy, 2004). Positively charged ions, such as sodium (Na^+) raise pH and negatively charged ions, such as chloride (Cl^-) lower pH. It is hypothesized that acidosis is an imbalance in acid-base that can be caused by low levels of cations associated with a starch-based diet (Mueller et al., 2001). Starch-based diets are generally low in DCAD compared to forages. Although energy digestibility has shown no variation in respect to DCAD, it may be possible to reverse metabolic acidosis caused by high starch intake by increasing the DCAD of the diet. Interestingly, urine pH is directly correlated with DCAD. Urine pH has been found to be lower in horses consuming diets with a low DCAD (Mueller et al., 2001). Furthermore, horses supplemented with grain appear to have a lower mean urine pH compared to the urine pH of grazing horses, and fecal pH appears to follow this same trend (Wood et al., 1990; Hussein et al., 2004; Harlow et al., 2016). Diet and exercise may affect each of these mechanisms regulating gastrointestinal pH to different degrees and in different ways.

1.2.3 Factors Affecting Gastrointestinal pH

Horses evolved consuming small, frequent meals of high-fiber forages throughout the day, making it unlikely that the stomach was ever empty (Menzies-Gow and Wray, 2019). However, the stomach may become relatively empty in modern feeding practices, which usually consist of two large concentrate meals and intermittent access to forage. Parietal cells in the lower, glandular region secrete HCl continuously, regardless of the amount of food in the stomach (Murray and Eichorn, 1996). Unlike the glandular region which secretes mucus and bicarbonate as a protective mechanism against the low pH of the acid produced, the non-glandular region does not possess this ability. Therefore, modern feeding practices paired with the high intensity exercise of performance horses can pose health risks. Intense exercise is associated with increased intra-abdominal pressure and gastric compression, pushing acidic contents onto the non-glandular region of the stomach (Lorenzo-Figueras and Merritt, 2002). If the stomach is relatively empty, a greater portion of the non-glandular region is at risk of acid splashing.

Furthermore, the common practice of feeding two distinct concentrate meals also poses a risk of overwhelming the digestive abilities of the small intestine with excess NSC (Potter et al., 1992). Larger meals tend to pass through the gastrointestinal tract faster compared to the smaller, more frequent meals the horse evolved to consume (Clarke et al., 1990). Therefore, feeding two large grain meals a day likely increases the rate of passage of NSC, contributing to the likelihood of reaching the hindgut undigested (Drogoul et al., 2001).

Exercise-induced blood flow redistribution may also affect rate of passage as blood is diverted from the gastrointestinal tract to skeletal muscles (Pagan, 1998). This in turn may affect gastrointestinal pH. The downstream consequences of NSC reaching the large intestine undigested are discussed in the following section. Intense exercise may affect gastrointestinal pH by altering the equine microbiome (Almeida et al., 2016). Bacterial populations were characterized from fecal samples obtained before and after fillies underwent acute exercise. Intra-group comparisons showed intense exercise changed

microbial composition and structure. Further research is needed to understand how exercise-induced changes in microbial populations may influence gastrointestinal pH.

1.3 Influence of pH on Gastrointestinal Disease

Whether exercise-, nutritionally-, or stress-induced, pH changes contribute to development of diseases occurring within any section of the gastrointestinal tract. Two ways in which changes in pH can contribute to gastrointestinal disease include acidosis and ulceration.

Equine Gastric Ulcer Syndrome (EGUS) is a prevalent health issue potentially affecting up to 90% of racing Thoroughbreds in active training (Lamglait et al., 2017). The disease can be further categorized based on location of ulceration in the stomach which are likely caused by different mechanisms. Equine Squamous Gastric Disease (ESGD) is characterized as formation of ulcers in the non-glandular region of the stomach. Squamous ulcers are primarily due to prolonged exposure to gastric acids (Sykes et al., 2015). The modern feeding strategy of two distinct grain-based meals with decreased roughage intake can cause the squamous region to become inadequately protected from acids secreted in the glandular region. High-intensity exercise likely exacerbates the issue by increasing splashing of acids onto the squamous region. Equine Glandular Gastric Disease (EGGD) is characterized by ulcer formation in the lower, glandular region of the stomach. Glandular ulcers may be due to disruption of blood flow and decreased bicarbonate and mucus secretion. Gastric inflammation, termed gastritis, can be the result of bacterial infection that also contributes to gastric ulcer formation in humans (Bravo et al., 2018). It remains unclear whether bacteria are involved with the formation and/or persistence of gastric ulcers in horses (Banse and Andrews, 2019).

Starch intake greater than 3g per kg per body weight can overload small intestine digestion (Potter et al., 1992). Undigested starch and sugars enter the cecum where they are fermented by microbes into lactic acid (Biddle et al., 2013). The resulting decrease in pH can cause hindgut acidosis, which is

considered to be pH values below 6 (Radicke et al., 1991; Garner et al., 1978). Damaged epithelium and altered mucus production has been reported in cattle and swine following acidosis (Khafipour et al., 2009; Salzman et al., 1994). This can leave submucosal tissue vulnerable to the acidic lumen contents and potentially increases the risk of colonic ulceration. The compromised epithelial barrier may also increase gastrointestinal permeability (Biddle et al., 2013). "Leaky gut", a term for alterations in permeability, can cause the intestinal barrier to ineffectively prevent toxins from entering (Stewart et al., 2017). Additionally, endotoxins released from necroptosis of fiber-digesting microbes subject to the low lumen pH levels can compromise gastrointestinal health. It is hypothesized that laminitis may be initiated in response to hindgut-derived endotoxins following carbohydrate overload (Moore et al., 1979; Zerpa et al., 2005).

Increasing forage consumption and gradually reducing the amount of NSC fed can allow the microbiome to adjust and maintain epithelial integrity (Garber et al., 2020). However, diets relatively high in NSC may contribute to a low hindgut pH that may not be low enough to culminate in laminitis but contribute to subclinical acidosis, decreased forage digestibility, and performance. The ability to non-invasively and accurately measure changes in gastrointestinal pH would be valuable in identifying horses at risk or in the early stages of a gastrointestinal disease process.

1.4 Fecal pH as an Indicator of Gastrointestinal Health

A multidimensional approach is necessary to evaluate gastrointestinal health and potentially pinpoint the part of the tract where a disturbance has occurred. Measurement of fecal pH has been used as a simple and noninvasive tool to indirectly measure gastrointestinal changes in pigs and rats (Howard et al., 1995; Campbell et al., 1997). Equine nutrition studies commonly use fecal pH to monitor perturbations in hindgut pH following increased NSC consumption and changes in feeding strategies (Berg et al., 2005; Hussein et al., 2004). Whether fecal pH measurements accurately reflect the pH of the

hindgut or detect short-term changes in gastrointestinal pH are points of disagreement within the literature.

Fecal pH has been thought to be an indicator of cecal and/or colonic pH due to their proximity to the rectum (Hale and Thompson, 2007; Willing et al., 2009). Differences in fecal pH between horses could be a reflection of differences in production and/or absorption of individual VFAs from the hindgut (van Soest, 1994). However, studies specifically investigating a correlation between cecal and fecal pH have not consistently supported this notion. Short term fluctuations in cecal pH following a single meal were not detected by fecal pH in a study by Næsset and Austbø (2010). Douthit et al. similarly found that fecal pH appeared to have limited use in predicting cecal pH using a prediction equation, potentially due to differences in transit time of digesta from the cecum to colon (2014). An association was found between minimal cecal and fecal pH. Graß states that pH values of fecal samples were not equal to digesta from any part of the hindgut, but rather increases in cecum and colon pH were mirrored by increases in fecal pH (1995). While pH values of the hindgut are highly dependent on the amount of time since the last meal was fed, changes in fecal pH appear to be more stabilized. Willing et al. found no significant difference in fecal pH measured on the last day of a 29-day experimental period between horses fed a high-energy forage-only diet or forage-concentrate diet in a cross-over design (2009). Early and/or subtle changes in fecal pH may have been detected if measurements were taken throughout the experimental period (Willing et al., 2009). Further research investigating the ability of repeated measurements throughout the day to detect fluctuations in fecal pH within horses in response to meals could be beneficial.

The ability of fecal pH measurements to detect differences between treatment groups is likely largely dependent on the diets being compared and whether comparisons are being made between or within groups. There is potentially an increased likelihood of detecting a difference in fecal pH between horses fed two diets drastically different in SC and NSC concentrations. Furthermore, there may be an increased likelihood of detecting fecal pH differences when comparing different diets fed to the same

horse. Muhonen et al. found that colon and fecal pH were unchanged after abruptly transitioning horses from grass hay to grass silage or grass haylage fed at similar dry matter intakes (2009). van den Berg et al. found fecal pH values initially increased when abruptly transitioning pasture grazed horses to confinement for a 13-day period of concentrate feeding in monitoring development of clinical acidosis (2013). By day five, fecal pH had decreased to pasture baseline values and no significant difference in mean pH was found during feeding an increased concentrate to forage (C:F) ratio days 5-13 (van den Berg et al., 2013). Regression showed an increased C:F ratio negatively affected pH with up to a 1-day lag. Furthermore, a positive relationship was found between increasing bacterial numbers of *Streptococcus spp* and *Lactobacillus spp* and ratio of C:F. This suggests measuring bacterial counts in addition to fecal pH could be useful when monitoring the effects of dietary transition on hindgut acidosis.

In support of meal-dependent fecal pH changes, the pH values of fecal samples collected before and after a 16-hour period of feeding ad libitum hay were found to be significantly different ($P<0.001$) by Hale and Tompson (2007). Furthermore, fecal pH has been shown to significantly decrease following large loads of NSC administered to induce laminitis (Pollitt and Davies., 1998). A sharp decline in fecal pH was detected in all horses 16 hours after carbohydrate administration. However, the rapid fall in fecal pH was not significantly different between the six laminitic positive and eight laminitic negative horses. All in all, measuring fecal pH as an indicator of gastrointestinal change may be most valuable when measured regularly within horses and in conjunction with other methods such as bacterial population counts (Milinovich et al., 2008).

The reason for gathering fecal pH measurements should be considered when determining the importance of the accuracy and precision of the pH measurement protocols used. Accuracy and precision are extremely important in research using fecal pH to detect differences between treatment groups and in a clinical setting, such as the diagnosis of acidosis. As described in the section above, fecal pH may not accurately reflect the pH of the gastrointestinal tract and therefore should be used in addition to or

replaced by other techniques when a more definitive diagnosis is required. Evaluating fecal pH as well as blood and urine pH may be more useful when assessing the severity of disease and as an indicator of when to pursue further diagnostic testing (Bohn, 2013). However, accuracy and precision may not be as essential in the practical setting of a horse-owner measuring fecal pH to acquire a baseline value, identify differences following a diet chain, or corroborate suspicion of gastrointestinal disturbance requiring a veterinarian's assistance.

1.5 Fecal pH Analysis Protocols

The conventional protocols used to measure fecal pH are relatively simple and straightforward. The process involves collecting, storing, and preparing the fecal samples for obtaining pH measurements. Differences in approaches used or lack of clarity describing the approach used threaten the replicability and credibility of prior research findings. Standardizing each of these steps can improve precision and accuracy of fecal pH measurements and allow for more productive between-study comparisons. This section discusses variance in the current protocols used to analyze fecal pH, outlines the ideal approach for each step based on the literature, and emphasizes areas of improvement.

1.5.1 Fecal Sample Collection and Storage

The first step in analyzing fecal pH is collecting fecal samples. It is unclear whether fecal pH can detect fluctuations in response to a single meal. Differences in diet and exercise contributing to variability in rate of passage may explain this disagreement. The best practice may be to collect fecal samples before the first morning feeding to avoid potential meal-induced changes on fecal pH and ensure repeatability of results. Samples collected directly from the rectum may be the most accurate method as there may be variance in pH between fecal samples collected from the rectum and from the ground shortly after

defecation (Næsset and Austbø, 2010). Fecal samples in the present study were collected from the ground due to animal welfare risks.

The storage technique used between collection and preparation of fecal samples for pH measurement is ambiguously described within the literature. Some studies detail using fresh samples but do not include the amount of time between collection and preparation of the sample (Hale at al., 2007; Milinovich et al., 2007). Other studies describe freezing fecal samples for an undefined amount of time before measuring the pH (van den Berg et al., 2013; Zeyner et al., 2004). Variance in storage technique has the potential to influence fecal pH measurements and is a part of what drove the objectives of this study.

1.5.2. Fecal Sample Preparation

Using fecal liquid only as opposed to diluting the sample with distilled water yields the most accurate pH meter measurements, permitting the sample has a high enough moisture content to submerge the entire electrode in the fecal liquid (Hydock et al., 2014). The addition of distilled water has been found to increase the pH of fecal samples ($P < 0.05$) as similarly observed in soil analysis (Hydock et al, 2014; Hendershot et al., 2007). Cheesecloth can be used to strain the fecal sample to obtain solely liquid. If the addition of distilled water is necessary to obtain enough liquid to fully submerge the meter electrode, the mixture should be stirred for 3-4 minutes before measuring pH to ensure full homogenization (Hydock et al., 2014).

1.5.3. Methods of Fecal pH Measurement

The pH meter is the standard tool for measuring the pH of fecal samples. To obtain the most accurate readings, the pH meter should be calibrated using at least two calibration points before

measuring fecal pH (Hydock et al., 2014). Once the meter electrode is fully submerged in the stirred sample, the pH measurement should be recorded after the reading has stabilized. A portable pH meter can range in price from $300 to $500. This can be costly for some, limiting certain demographics from using this pH measurement method. Little to no research has been conducted regarding the accuracy and precision of other tools in measuring fecal pH.

One potential alternative pH measurement tool is the soil pH meter. Before conducting the present study, we attempted to measure fecal pH with two soil pH meters: the Rapitest Electronic Soil Tester (Luster Leaf 1860) and the Moisture, Light & pH Meter (HoldAll 60182L). Neither soil meter was able to pick up pH readings of the fecal samples. Another prospective tool is pH strips which could serve as a simple, inexpensive, and non-invasive measurement technique. A pH strip is a strip of litmus paper that turns different colors to reflect the acidity of the liquid being tested. However, pH strips are likely inherently less accurate than the portable pH meter because of the smaller range in pH certain strips can detect as well as the pH increments of the strips. This should be kept in mind in addition to the purpose of measuring fecal pH when determining which measurement tool to use.

1.6 Summary

The pH of the equine gastrointestinal lumen influences digestion, the microbiome, and enterocyte physiology, each having important impacts on equine health and performance. Many factors including diet and exercise can alter gastrointestinal pH, potentially increasing the risk of disease. Measuring fecal pH offers potential insight into evaluating gastrointestinal health. Standardizing the protocol used to analyze fecal pH could improve accuracy and precision of pH measurements and enable the detection of differences between treatment groups. The purpose of the study was twofold: (1) to evaluate whether there is agreement between fecal pH measured by a meter and pH strips and (2) to determine the effect of storage temperature and time on fecal pH.

Chapter 2

Evaluation of Equine Fecal pH Measurement Methods and Storage

2.1 Introduction

Modern feeding and housing strategies as well as the athletic requirements of performance horses differ from the conditions in which the horse evolved. As a result, the gastrointestinal tract is subject to disturbances in pH. This can increase the risk of health conditions including ulceration, inflammation, and acidosis. Fecal pH is a simple and noninvasive variable that may serve as an indirect indicator of gastrointestinal health by reflecting changes in hindgut pH. Variation in the protocols used to measure fecal pH hinders productive between-study comparisons and the ability to detect differences between treatment groups.

The conventional protocol used to measure fecal pH involves four main parts: collection, storage, preparation, and measurement. Collection involves the process of obtaining feces. Two common practices are collecting feces directly from the rectum or immediately after defecation. Next, fecal samples can be either immediately prepared for pH measurement or stored via refrigeration or freezing. In preparing fecal samples for analysis, prior studies have diluted fecal samples with distilled water before measuring pH. Hydock et al. (2014) found that distilled water influenced fecal pH measurements, so fecal liquid alone should be used, when possible, to avoid interference. A hand-held pH meter is the standard tool used to measure fecal pH. However, pH strips could serve as an alternative, inexpensive, and simple measurement tool. This study focused on potential sources of error regarding storage and measurement techniques.

Our hypothesis was that pH strips accurately and precisely reflect pH meter measurements, and the pH of refrigerated and frozen fecal samples is not significantly different from pH of fresh fecal samples. The first objective was to evaluate whether there is agreement between fecal pH measured by a

pH meter and pH strips. The second objective was to determine the effect of storage temperature and time on fecal pH.

2.2 Materials and Methods

2.2.1 Horses and Management

This study was performed between the months of April and November 2022 at the Penn State University Park campus and Kocher Equestrian Center in State College, Pennsylvania. Fecal samples were collected from horses of varying housing conditions, feeding strategies, and disciplines to evaluate if pH strips could accurately and precisely detect a large range of pHs. Fecal sample collection was divided into four collection days: April 10th, April 24th, June 12th, and July 17th. On April 10th, seven fecal samples were collected from Penn State Quarter horses: one broodmare, two gestating mares, and four yearlings (Table 1). On April 24th, six samples were collected from different Penn State Quarter horses: four two-year-old's and two dams (Table 1). Seven fecal samples were collected on June 12th and five on July 17th from Kocher Equestrian Center horses: seven hunter/jumpers, two retirees, and three recreationally ridden (Table 2). The horses used for sample collection range in age, diet, housing condition, exercise program, and use (Tables 3 and 4). Use was divided into four categories: breeding, non-working, recreation, and Hunter/Jumper. Breeding denotes horses that were currently in gestation or foaled within the year. Non-working indicates the horse was not currently exercised/in a training program or was retired. Recreation indicates the horse was irregularly exercised, and Hunter/Jumper indicates the horse was consistently exercised in a structured training program.

Table 1. Details of the Penn State Quarter horses.

Horse	Age (Years)	Sex[1]	Use
1	11	m	Breeding
2	15	m	Non-working
3	14	m	Breeding
4	1	g	Non-working
5	1	m	Non-working
6	1	g	Non-working
7	1	m	Non-working
8	2	m	Recreation
9	2	m	Recreation
10	2	g	Recreation
11	2	m	Recreation
12	21	m	Breeding
13	11	m	Breeding

[1]Sex m denotes a mare, and g denotes a gelding.

Table 2. Details of the Kocher Equestrian Center horses.

Horse	Age (Years)	Sex[1]	Use
14	5	g	Hunter/Jumper
15	24	g	Hunter/Jumper
16	27	g	Recreation
17	23	g	Non-working
18	22	g	Recreation
19	19	g	Hunter/Jumper
20	16	g	Hunter/Jumper
21	21	g	Recreation
22	25	g	Hunter/Jumper
23	12	g	Hunter/Jumper
24	18	g	Non-working
25	16	g	Hunter/Jumper

[1]Sex m denotes a mare, and g denotes a gelding.

Table 3. Management details of the Penn State Quarter horses.

Horse	% turnout/week	Amnt. conc. fed[1] (kg/day)	Method of conc. feeding	Type of hay fed
1	29	3.2	Individually	g[4]/a[5]
2	100	3.2	Group fed[2]	g
3	29	3.2	Individually	g/a
4	100	2.4	Group fed[3]	g
5	100	2.4	Group fed[3]	g
6	100	2.4	Group fed[3]	g
7	100	2.4	Group fed[3]	g
8	29	3.2	Individually	g/a
9	29	3.2	Individually	g/a
10	29	3.2	Individually	g/a
11	29	3.2	Individually	g/a
12	29	6.4	Individually	a
13	29	6.4	Individually	a

[1]Amount concentrate fed in kg/day
[2]Group fed with own individual feed bucket
[3]Group fed with shared feed buckets
[4]Grass hay fed
[5]Alfalfa hay fed

Table 4. Management details of the Kocher Equestrian Center horses.

Horse	% turnout/week	Amnt. conc. fed[1] (kg/day)	Method of conc. feeding	Type of hay fed
14	25	2.7	Individually	g[2]
15	11	8.2	Individually	g
16	11	5.4	Individually	g
17	18	5.4	Individually	g
18	25	5.4	Individually	g
19	18	8.2	Individually	g
20	25	8.2	Individually	g
21	18	2.0	Individually	g
22	14	5.4	Individually	g
23	14	5.4	Individually	g
24	11	8.2	Individually	g
25	11	5.4	Individually	g

[1]Amount concentrate fed in kg/day
[2]Grass hay fed

2.2.2 Experimental Design

Horses were observed free of disruption during fecal sample collection. Fecal samples were collected between 8:00am to 12:00pm from individual horses within one minute of defecation. Particular attention was given to ensure that fecal samples were obtained from an uncontaminated region of the pile. Approximately a half to a full-sized gallon bag of feces was collected for each sample. Fecal samples were then placed in a sealable plastic bag and immediately put on ice for transportation to the laboratory for analysis. Samples were either analyzed or processed for storage within approximately three hours of defecation. Once in the laboratory, fecal samples were homogenized by hand and divided into four sub-samples to measure pH at different storage timepoints and temperature: fresh, 24-hour refrigerated at 4° C, 6- and 16-weeks frozen at -20° C. A calibrated hand-held pH meter (Hanna Instruments HI98121 Waterproof pH/ORP/Temperature Combo Meter; Smithfield, RI) and three pH strips were used to measure fecal pH. The ranges of the three pH strips (VWR Chemicals BDH®) were 4.5-10 (Strip 1), 5.1-7.2 (Strip 2), and 6.0-7.7 (Strip 3). Strip 1 increased in increments of 0.5, and Strip 2 increased in increments of 0.3. Strip 3 increased from 6.0-6.4, then increments of 0.3 from 6.4 to 7.3, and from 7.3-7.7.

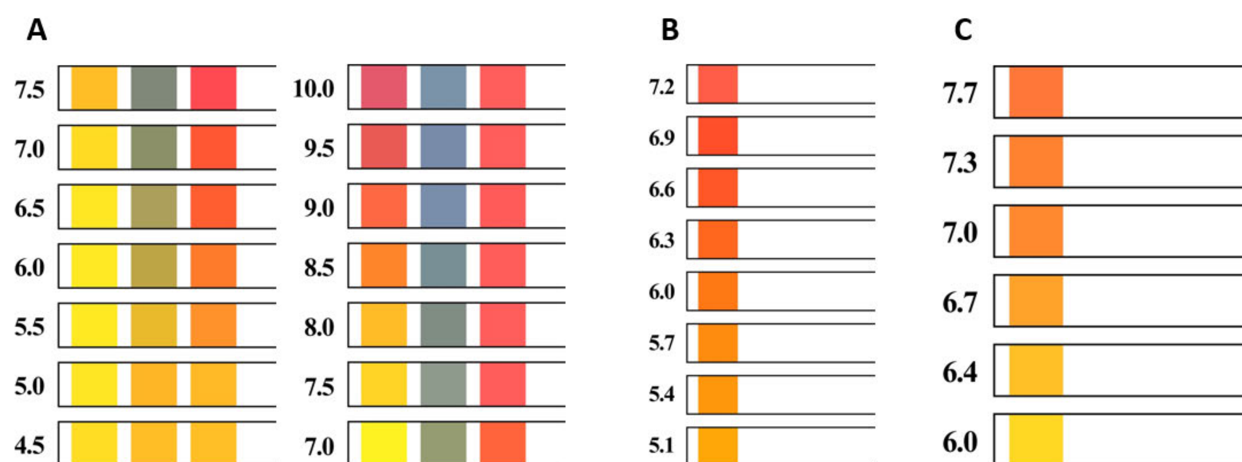

Figure 1. Ranges of the three pH strips used: (A) Strip 1, (B) Strip 2, and (C) Strip 3.

2.2.3 Sample Analysis

Fecal pH was measured using a recent protocol that optimizes accuracy and precision (Hydock et al., 2014). Prior to pH analysis, refrigerated and frozen samples were placed in a water bath at 20-23° (room temperature) and allowed to thaw until the water stabilized at 20-23° C. Water was replaced periodically to accelerate the thawing process. Refrigerated samples took approximately one hour and frozen samples two and a half hours for the water bath to stabilize at 20-23° C. The pH meter was calibrated with buffered solutions of pH 4 and 7 before each analysis session.

The steps described below were used to measure the pH of all sub-samples. Fecal samples were re-homogenized for approximately one minute and squeezed in the center of a piece of cheesecloth two layers thick to obtain between 25mL and 40mL of fecal liquid in a 150mL glass beaker. A mixture of fecal liquid and distilled water was used for strip analysis due to staining that rendered strips unreadable when using fecal liquid alone. A 1mL plastic test tube was filled with 1/3 fecal liquid and 2/3 distilled water for a 1:2 ratio. The tube's top was covered with parafilm and inverted five times to mix fecal liquid and distilled water. Parafilm was removed and three Strip 1 strips were submerged into the tube. The ends of the strips were twisted to ensure each was fully saturated and then immediately removed for comparison against the key on the Strip I package. Two observers recorded their measurements of the three pH strips side-by-side, without observing or conferring with one another. Three strips of Strip 2 and then Strip 3 were similarly submerged, read against the key, and separately recorded by the observers.

A small magnetic stir rod was placed into the beaker with the remaining fecal liquid and put onto a magnetic stir plate at a medium-high setting for 3 minutes. The beaker was removed from the stir plate after 3 minutes and the meter electrode was fully submerged into the fecal liquid. Upon stabilization of the pH reading, temperature and pH measurement were recorded. The beaker was returned to the stir plate while the pH meter electrode was rinsed thoroughly with distilled water. The electrode was carefully

dried with a clean paper towel before taking the next measurement. A total of three readings were recorded with the pH meter within 6 minutes of stirring.

2.2.4 Statistical Analysis

Stata software, version 17.0 (Stata Corp LLC; College Station, TX), was used to analyze all data. A coefficient of variation (CV) was determined for the multiple meter readings and for the strip readings. All measurement techniques had CV's below 10%. All reported pH measurements represented pooled averages of technical replicates. A paired t-test was used to identify differences between the pH meter measurements and pH strips. Linear regression was used to assess the relationship between meter and strip pH measures, and Bland-Altman plots were used to quantify agreement between the pH meter and strips. Both the linear regression and the Bland-Altman plots provided indicators of accuracy and precision of the strip pH measures. A mixed ANOVA was conducted to evaluate the fixed effect of storage conditions on measured pH. Horse and farm were included in the mixed model as random effects. The four storage conditions were (1) fresh, (2) 24-hour refrigerated at 4° C, (3) 6-weeks frozen at -20° C, and (4) 16-weeks frozen at -20° C. Post-hoc comparisons of the storage conditions were computed with a Bonferroni correction to decrease the likelihood of false-positives or type I errors. Difference was determined to be significant at $P \leq 0.05$ and a trend at $P \leq 0.01$. Data are presented as means±sem.

2.3 Results

2.3.1 pH Meter vs Strips

The pH meter was used as the gold-standard when determining the accuracy and precision of the pH strips. The CV for fecal pH measured by the pH meter was 0.5%. The CV for fecal pH measured by

strips was 1.3% for Strip 1, 1.3% for Strip 2, and 1.1% for Strip 3. These low CVs indicate excellent repeatability of technical replicates for all techniques. Paired t-tests indicated that pH as measured by Strips 2 trended lower and 3 was lower than that measured by the meter (Table 5).

Linear regression indicated that strip 2 had the highest r^2, with a y-intercept closest to zero and a slope close to 1 (Figure 2). All three of these characteristics indicate that strip 2 had the highest accuracy and good precision. Strip 1 was the next closest, in regard to the y-intercept and slope, but the lower r^2 indicates poorer precision. Finally, strip 3 had an r^2 between strip 2 and 1, but a poor y-intercept and slope that indicates poor accuracy and a bias in the data as pH rises. Strip 1 had the highest root mean square error (RMSE) of 0.22, indicative of high variance and low precision in predicting pH meter measurements. Strip 3 had the lowest RMSE (0.15) that was similar to the RMSE of Strip 2 (0.19).

Bland-Altman plots were used to quantify and visualize agreement between the pH meter and strips. The differences between fecal pH measurements were expressed against the average of the two measurements. The Bland-Altman plot analysis comparing Strip 1 and the pH meter showed the smallest mean bias (0.005), indicating that there was very little difference between the average of Strip 1 and the pH meter. The 95% limits of agreement were -0.45 to 0.46 (Figure 3-A). The mean difference of the linear trend line was 0 which indicates that there was no relationship between the value of fecal pH and the difference between Strip 1 and pH meter measurements (Figure 4-A). Agreement limits slightly enlarged as the average of the pH meter measurements and Strip 1 increased.

The Bland-Altman plot analysis comparing Strip 2 and the pH meter had a negative mean bias (-0.03), indicating Strip 2 gave lower averages than the pH meter. The 95% limits of agreement showed the least spread of all Bland-Altman plots from -0.41 to 0.34 (Figure 3-B). The plot showed a moderately positive trend between the difference and the average of Strip 2 and the pH meter (Figure 4-B). This indicates that as fecal pH increased, the difference between Strip 2 and the pH meter tended to increase. The enlargement of the 95% limits of agreement as the average of Strip 2 and the pH meter increased suggests that the difference between the two measurements is not constant across all fecal pH values.

The Bland-Altman plot analysis comparing Strip 3 and the pH meter also showed a negative mean bias of -0.04 and 95% limits of agreement from -0.43 to 0.34 (Figure 3-C). The plot showed a negative trend between the difference and average of Strip 2 and the pH meter (Figure 4-C). This indicates that as fecal pH increased, the difference between Strip 2 and the pH meter tended to decrease.

Table 5. Summary of statistical values indicating agreement and bias between the pH meter and strips.

Device	Mean ± SD pH	95% confidence interval	*P* value
Meter*	6.46 ± 0.45	6.37-6.56	NA
Strip 1	6.47 ± 0.46	6.37-6.56	0.83
Strip 2	6.43 ± 0.51	6.33-6.53	0.09
Strip 3	6.42 ± 0.36	6.34-6.49	0.03

*Reference method
NA=Not applicable

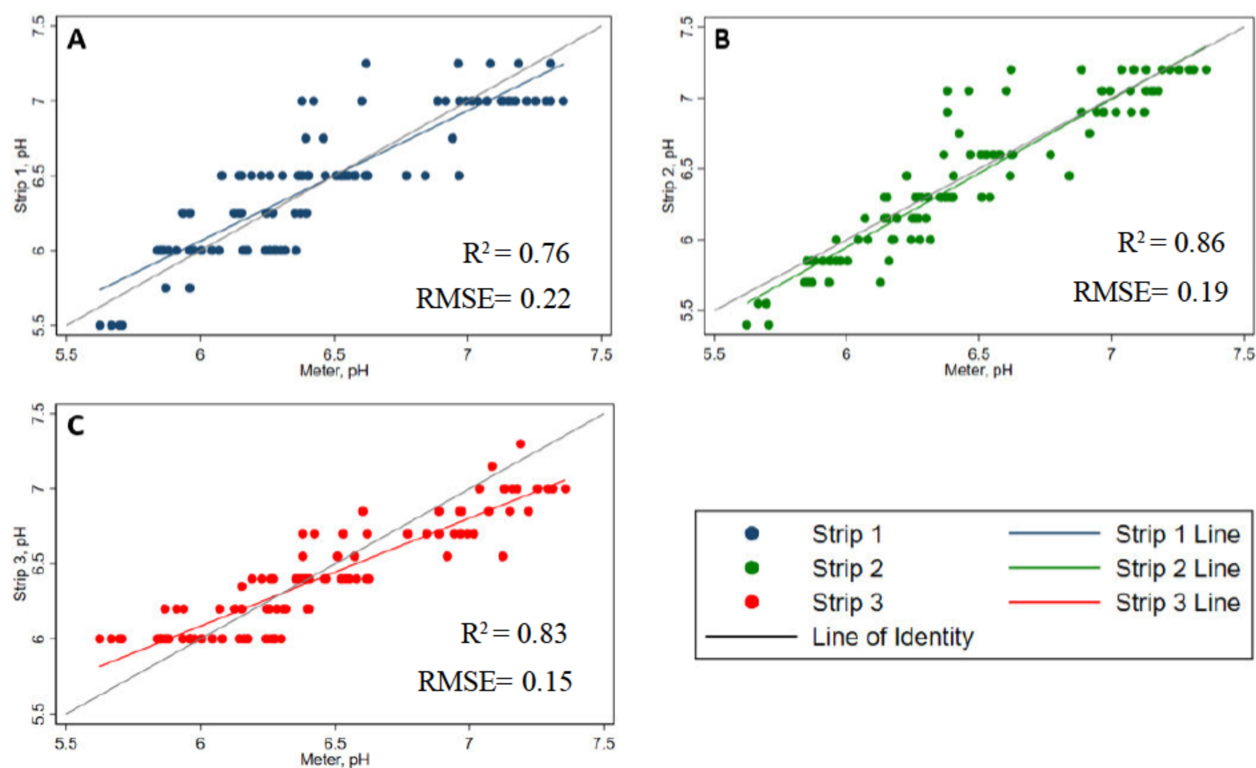

Figure 2. Linear regressions of the pH meter against Strips 1, 2, and 3. Regression analysis depicting the association between pH meter measurements and Strip 1 (A), Strip 2 (B), and Strip 3 (C). The fitted linear regression models are (A) Strip 1 = 0.83 + 0.87x, (B) Strip 2 = -0.32 + 1.05x, (C) Strip 3 = 1.78 + 0.72x, where x was fecal pH measured by the pH meter.

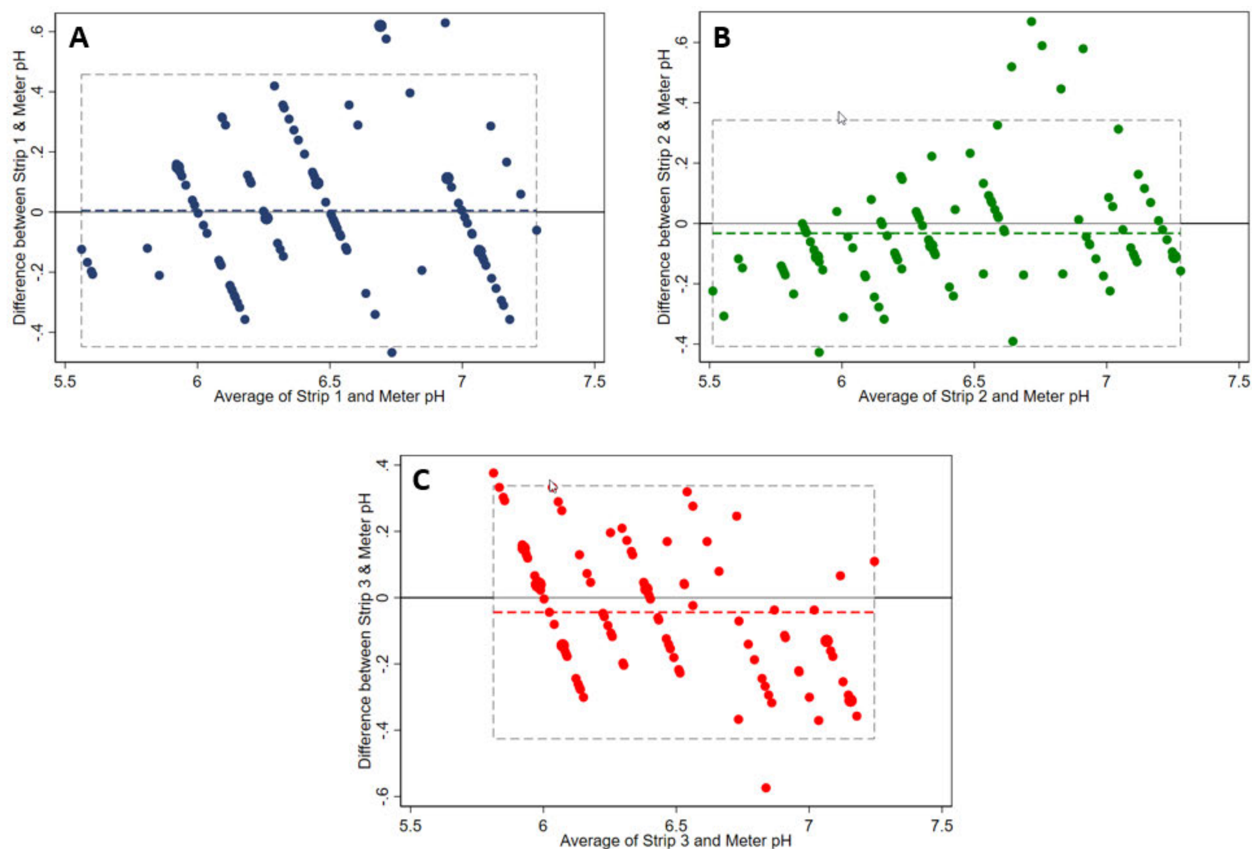

Figure 3. Bland-Altman plots depicting agreement between pH meter and strip readings. (A) The 95% limits of agreement (LOA) were between -0.45 and 0.46 with 5.0% of samples outside of the LOA. The mean difference was 0.005. (B) The 95% LOA were between -0.41 and 0.34 with 6.0% of samples outside of the LOA. The mean difference was -0.03. (C) The 95% LOA were between -0.43 and 0.34 with 2.0% of samples outside of the LOA. The mean difference was -0.04.

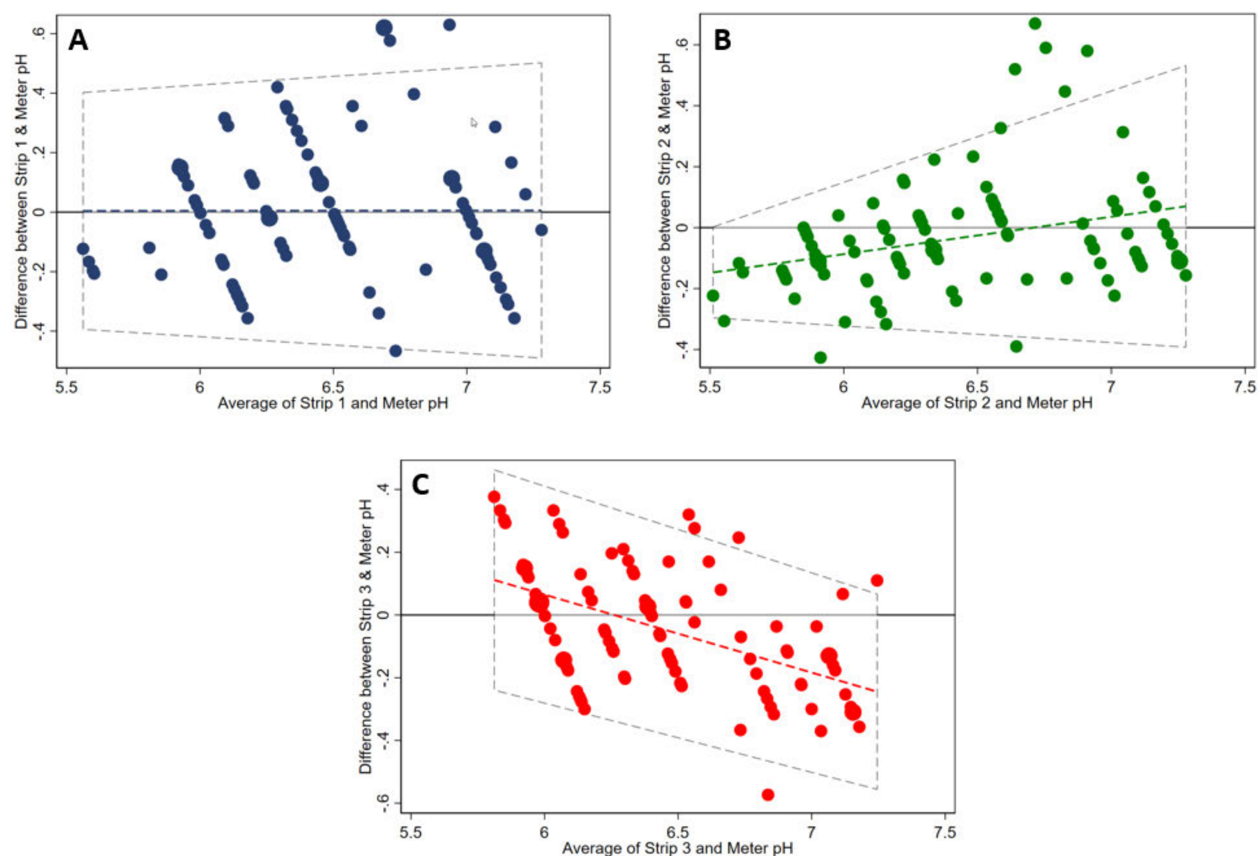

Figure 4. Bland-Altman plots with linear trend lines depicting agreement between pH meter and strip readings. (A) The 95% limits of agreement (LOA) were ± 2.46(0.03 + 0.02*Average) with 5.0% of samples outside of the LOA. The mean difference was -0.00 + 0.00*Average. (B) The 95% LOA were ± 2.46(-0.34 + 0.07*Average) with 9.0% of samples outside of the LOA. The mean difference was -0.82 + 0.12*Average. (C) The 95% LOA were ± 2.46(0.21 + -0.01*Average) with 5.0% of samples outside of the LOA. The mean difference was 1.56 + -0.25*Average.

2.3.2 Storage Temperature and Time on pH Measurements

One-way ANOVA indicated there was a significant effect of storage condition on fecal pH. The pH of the 24-hour refrigerated samples (6.39 ± 0.31) were significantly lower than both the fresh (6.49 ± 0.31) and 6-week frozen samples (6.47 ± 0.31, $P<0.001$). No statistically significant difference was detected in mean pH measurements between the 16-week frozen samples (6.43 ± 0.31) and the other sub-samples. However, there was a trend for the 16-week frozen samples to be lower than the fresh samples ($P = 0.082$).

Through analysis of the data, it became clear that there were noteworthy differences in the fecal pH of samples collected on the two farms included in this study. While not a primary objective, the effect of farm was evaluated further with ANOVA, examining the effects of both storage condition and farm as fixed effects. The fresh fecal samples were consistently lower on farm 2 versus farm 1 ($P<0.001$). The pH of Farm 1 (6.78 ± 0.11, 95% CI = 6.6-7.0) was higher than Farm 2 (6.20 ± 0.09, 95% CI = 6.0-6.4) as measured by the pH meter ($P < 0.001$; Figure 5).

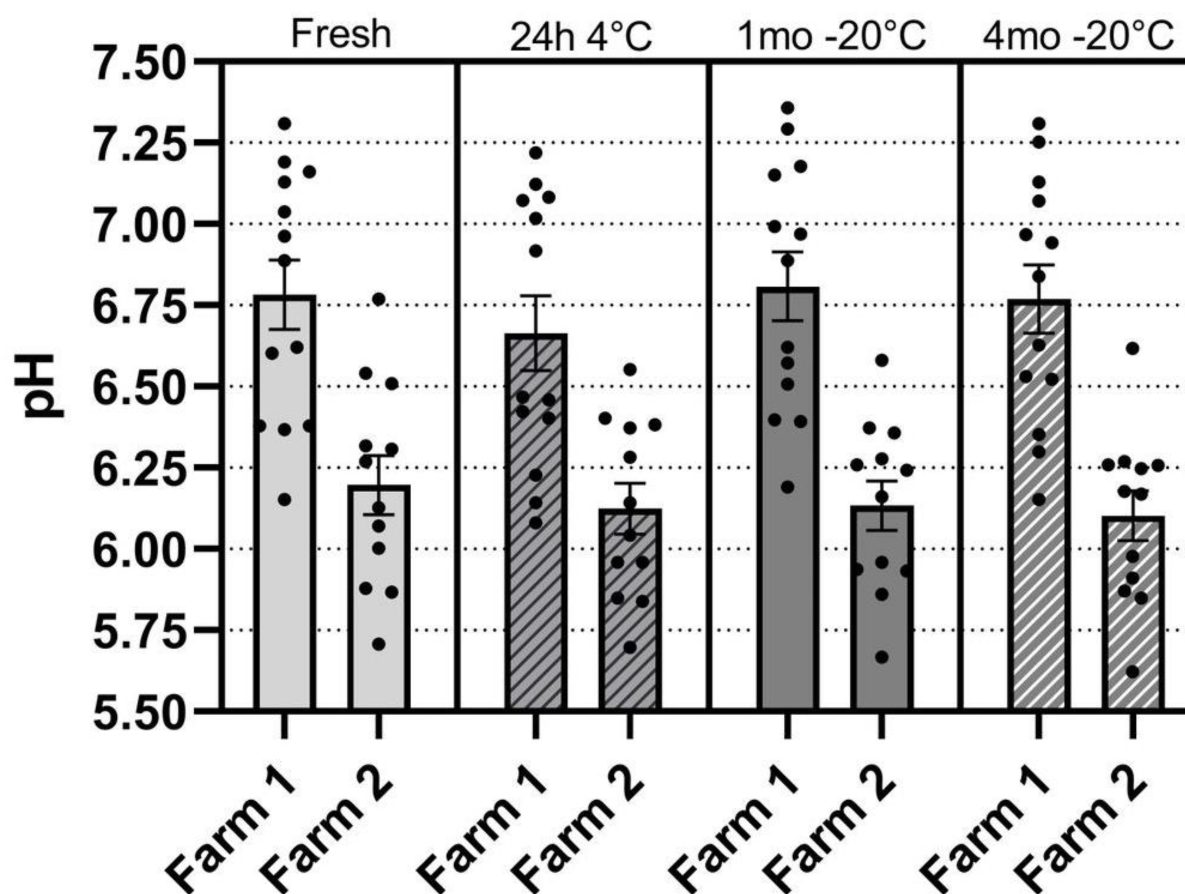

Figure 5. Comparison of meter fecal pH, farm, and storage protocol. The pH meter measurements of fresh, 24-hour refrigerated at 4° C, 6- and 16-weeks frozen at -20° C fecal samples from Penn State Quarter horses (Farm 1) and Kocher Equestrian Center (Farm 2).

2.4 Discussion

The three most important findings from this study are that storage conditions influenced measured fecal pH, Strip 1 was the most accurate and Strip 2 the most precise in reflecting pH meter measurements. This study was also able to detect a significant farm difference which may have been due to many reasons discussed below.

The pH meter measurements of 24-hour refrigerated samples were significantly lower than the fresh samples. However, pH meter measurements of the 16-week frozen samples were not found to be

significantly different from fresh or the 24-hour refrigerated samples. The trend for fecal pH measurements to decrease as the length of frozen storage time increased may indicate relevant change due to continued fermentation by microbes within the feces. Although the differences were small, there is value in the ability to detect differences which may be applicable with long-term frozen storage. Therefore, the best practice may be to measure fecal pH of fresh feces immediately after defecation.

Strip 1 most accurately reflected pH meter readings of all fecal pH values. The paired t-test detected no significant difference between Strip 1 and the pH meter, and Strip 1 had the smallest bias and a linear trend line of $y = 0$. This was most likely because Strip 1 had the widest range and largest pH increments. Strip 2 most precisely reflected pH meter readings, reflecting 86% of the variation in meter reading. Strip 2 also had the tightest limits of agreement on the Bland-Altman plots. The pH increments of Strip 2 were the smallest which could have contributed to its overall greater precision. Strips 2 and 3 were likely less accurate because their corresponding ranges were not wide enough to detect all of the fecal pH values measured by the meter. Because there were more fecal samples with pH values below Strip 3's range (pH of 6.0) than above Strip 2's range (pH of 7.2), Strip 2 was more accurate than Strip 3.

Furthermore, a 1:2 ratio of feces to distilled water was used for pH strip measurements to ensure readability related to color staining. The inclusion of distilled water could have contributed to the inaccuracy and imprecision of the strip readings as the addition of distilled water has been found to influence pH of fecal samples measured by a meter (Hydock et al., 2014). Although the fecal samples were diluted for strip measurement, staining of the strip indicators could have also influenced pH strip readings as well. pH strips may not be a suitable alternative to the pH meter in contexts where accuracy and precision are crucial. The ranges and increments of the strips used in this study were not able to accurately and precisely detect all fecal pH values compared to the pH meter. However, all bias values were within 0.5 pH units. Fecal pH is at best an indirect indicator of hindgut pH and the measurement alone cannot diagnose gastrointestinal disease. pH strips may give horse owners and veterinarians an adequate estimate of fecal pH and detect relevant differences within and between horses. Little to no

research has been conducted comparing the accuracy and precision of pH strips compared to the meter in measuring fecal pH as well as the effect of storage conditions on fecal pH measurements.

A farm difference in pH meter measurements was identified. Average fresh fecal pH of samples collected from Kocher Equestrian Center horses were significantly lower than the Penn State Quarter horse samples. All three pH strips were able to detect significant difference in fresh fecal pH measurements between farms. The reason for this difference is beyond the scope of this study although causes should be investigated in future research. Some relevant management details were recorded and could be implicated in the fecal pH difference found between farms (Tables 1-4). Examples of factors that may have led to this difference include greater overall concentrate intake on Farm 2, samples were collected from Penn State horses in April and from Kocher Equestrian Center horses in June/July, and the older aged horses sampled from Farm 2. This study did not control for these variables, nor was the experiment designed to investigate these differences. These variables may be of value to investigate in future work.

2.5 Conclusion

The results of this study indicate that pH strips overall were less precise and accurate than the pH meter and each strip differed in its ability to reflect pH meter readings. Strip 1 was the most accurate and least precise in reflecting pH meter measurements and Strip 2 was the most precise. The pH meter was able to detect differences in fecal pH between the storage protocols used in this study. The pH of the 24-hour refrigerated fecal samples were significantly different than the fresh and 6-weeks frozen samples. The 16-weeks frozen pH was not significantly different from any other timepoint. There may be a relevant trend towards a difference in the pH of stored fecal samples as length of frozen storage time increases.

pH strips may be useful in detecting relevant differences in fecal pH. When used to measure fecal pH on a consistent basis, pH strips may serve as an additional tool to evaluate changes in gastrointestinal health. The ability to detect fecal pH differences could be valuable in determining the best management strategy for optimal gastrointestinal health and to help reduce the occurrence of gastrointestinal disease. Fecal samples are commonly stored before measuring the pH as a response variable in equine nutrition studies. Storage temperate and length of storage may influence fecal pH. Therefore, measuring the pH of fresh fecal samples may contribute to the ability to detect relevant differences in pH.

Further research is needed to determine how well fecal pH reflects hindgut pH and its use in evaluating gastrointestinal health. Using an experimental model to standardize variables such as sex, age, turnout, and diet may be beneficial to understanding the effect of management strategy on fecal. Studies investigating the ability of pH strips to detect relevant differences in fecal pH will help determine its use in identifying horses at risk for disease. In determining its usefulness, pH strips may serve as an accessible simple, inexpensive, and non-invasive method to indirectly monitor gastrointestinal health.

APPENDIX

Equine Fecal pH SOP

Electrode Care & Maintenance:

- Clean **monthly** by submerging the electrode in H1700601 pH/ORP Electrode Cleaning Solution for 30 mins.
  - After the electrode is cleaned, rinse it thoroughly with distilled water.
- When not in use, add a few drops of H170300 Storage Solution to the protective cap.
- Calibrate with FRESH buffer solution before each use.
  - Use 2-point calibration for improved accuracy (pH 4.01 and 7.01 buffers).

Calibration Procedure:

- ***NOTE (Maintenance):*** *Calibration should be done once a month, or when electrode is replaced.*

1. Label 2 150 mL glass beakers for the pH 7.01 and 4.01 buffer solutions.
2. Pour 25 mL of the FRESH buffer solution into their corresponding beaker.
3. Turn on the pH meter and allow it to go into measurement mode.
4. Press and hold "MODE" for 3 sec. The device will say "OFF," but continue holding down the "MODE" button until "CAL" appears on the screen (it will be blinking).
5. Once the "CAL" is displayed, release the "MODE" button.
6. When the "pH 7.01 USE" message is displayed, place the electrode in the pH 7.01 buffer solution so that it is completely submerged in the solution.
7. Once meter is calibrated to 7.01 buffer solution, "REC" message will be displayed, followed by the "pH 4.01 USE" message.
   - If buffer was not valid, "WRNG" will be displayed. Remove electrode from buffer solution and resubmerge.
8. Next, place electrode in pH 4.01 buffer solution so that it is completely submerged and wait for the "REC" and "OK 2" message to be displayed.
9. When calibration procedure is completed, the "CAL" tag will stay turned on.

Preparing Fecal Samples for Analysis:

1. Obtain 3-4 frozen fecal sample(s) to be analyzed from the specific collection day, placing each of them in a second Ziplock bag.
2. Fill a small container with room temperature water and place a thermometer in the water bath.
   - Record the temperature.
3. Place 3-4 fecal samples in each water bath, recording the time that the samples were initially submerged.
   - ***IMPORTANT:*** *Ensure both Ziplocks are completely shut before submerging in the water bath!*
4. Allow samples to thaw until the temperature of the water bath reaches room temperature (20-23°C).
   - The water in the water bath may need to be replaced with fresh water periodically throughout the thawing process. Be sure to note the time this is done and the temperature of the fresh water.
   - ***NOTE:*** *Each batch of fecal samples takes approximately 2-3 hours to thaw to room temperature.*

5. Once the water bath has reached room temperature, remove 1 fecal sample and follow the "Fecal Sample Analysis Procedure," leaving the other 2 samples in the water bath until they are ready to be analyzed.
    - Be sure to record:
      i. the time that the water bath initially reached room temperature,
      ii. the time the sample was removed from the water bath for analysis,
      iii. and the temperature of the water bath when the sample was removed for analysis.
6. Repeat step 5 for each of the remaining samples.
    - ***NOTE:*** *Place the remaining 3-4 fecal samples from the specific collection day in a second water bath 1 hour after the first set. Do not allow samples to sit for extended periods of time before being analyzed (Remember: pH is temperature sensitive!).*

Fecal Sample Collection Procedure:

1. Only collect fresh fecal samples that you have witnessed the horse defecate (or know with 99% certainty that poop is new).
2. The scribe should begin recording details regarding the fecal sample on the analysis sheet: date and time of collection, name of horse, weather and where collected. Label Ziplock bag with horse name and date.
3. Take a picture of the fecal pile before picking it up.
4. Using a glove, collect feces from the center of the pile, limiting possible contamination from contact with the ground or foreign material and place it into a Ziplock bag. Be careful not to pick up hay, dirt, feces directly touching the ground but whilst collecting as much of the sample as possible.
5. Observe and record fecal characteristics: texture, color, consistency, particle size upon collecting the sample based on fecal scoring sheet.
6. Ensure Ziplock bag is fully sealed and place the sample on ice immediately.
7. Repeat steps 1-6 to collect the remaining 5-6 samples on each collection day.

Fecal Sample Analysis Procedure:

8. Calibrate the pH meter following the "Calibration Procedure" listed above before each analysis session.
9. Hand homogenize the fecal sample to be analyzed for approximately 1 minute, or until the sample seems like it is completely homogenized.
10. Divide the sample into 4 equal parts, placing 3 of the parts into separate, individual Ziplock bags labeled 24 hours refrigerated, 1 month frozen, 4 months frozen. Spread the fecal sample flat within the bag so that it thin and stretches across the bag entirely. Place the 3 bags into the –20 degree C freezer.
11. With the 4th unbagged part, carefully place the fecal sample in the center of a piece of cheesecloth (2 layers thick).
12. Squeeze the fecal sample and collect a minimum of 25 mL, but no more than 40 mL, of the resulting liquid in a 150 mL glass beaker.
    - A funnel may be used to help capture the fecal liquid.
13. Using a sterile pipette, gather enough fecal liquid to fill the plastic dilution tube to the first red line.
14. Using a separate sterile pipette, gather enough distilled water to fill the same plastic dilution tube to the second red line.

The following steps 15-18 and 19-26 must be executed simultaneously.

15. Secure parafilm over the plastic dilution tube and invert the tube five times, homogenizing the distilled water and fecal liquid.

16. Remove the parafilm, and dip 3 strips of pH 4.5-10 range into the plastic dilution tube, twisting the held ends to ensure each strip is fully saturated.
17. Immediately remove and compare the strips to the key on the back of the appropriate pH range package. 2 people should write down their observations of the pH on separate papers **without conferring**.
18. Repeat steps 9-11 with the pH 5.1-7.2 strips and pH 6.0-7.7 strips.
19. Place a small magnetic stir rod into the beaker with the remaining fecal liquid.
20. Place the beaker on a magnetic stir plate and stir liquid for 3 minutes.
21. While the fecal liquid is stirring, press "SET/HOLD" to select pH mode on electrode.
22. Once the fecal liquid has been stirred for 3 mins, remove sample from the stir plate and **fully** submerge the electrode in the sample.
23. Record the temperature and pH measurement on the pH meter after the stability tag (the icon that looks like a stopwatch) has disappeared. Also, be sure to record time (in minutes and seconds) that the measurement was taken.
24. Remove the electrode from the fecal liquid and return the beaker to the stir plate.
25. Rinse the pH meter thoroughly with distilled water, and carefully dry with a clean paper towel before taking the next measurement.
    - A total of 5 measurements will be recorded for each sample and the average reported.
    - All measurements should be obtained within a 3–5-minute period.
26. Once all 5 measurements are recorded, the fecal liquid can be poured down the sink drain. The beaker and magnetic stir rod should be rinsed with hot water, followed by distilled water, and dried prior to analyzing the next fecal sample. The electrode of the pH meter should be rinsed thoroughly with distilled water and carefully dried.
27. Repeat this procedure for each fecal sample that will be analyzed.
28. After analysis is completed:
    i. Return equipment to its proper storage place.
    ii. Clean benchtop and floor.
    iii. Wash glassware with soap and distilled water and place on drying rack.
    iv. Thoroughly rinse pH meter with distilled water and add a few drops of Electrode Storage Solution to pH meter cap for proper storage.
    v. All used gloves, cheesecloth, Ziplock bags, and fecal material may be disposed of in the regular trash, making sure to double bag it, and placed in the dumpster behind the ASI building.

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ACADEMIC VITA

Education

The Pennsylvania State University, University Park, PA
Schreyer Honors College '23
College of Agricultural Sciences - Veterinary and Biomedical Sciences Major & Equine Science Minor
Eberly College of Science - Neuroscience Minor

Work Experience

Penn State University - Neurobiology Class 469 State College, PA
Teaching Assistant August 2022 - December 2023
- Assisted Dr. Luscher with in-class questions, held weekly office hours & review sessions for each exam, helped students' develop study strategies, and explained missed exam questions

J M Ritchie Veterinary Hospital State College, PA
Veterinary Assistant May 2021 - August 2022
- Assisted with restraining animals during x-rays, ran fecal and urine analysis, monitored animals recovering from surgery lab, and medicated animals
- Interacted directly with clients by answering the phone, scheduled appointments, took payments

Ansley Animal Clinic Atlanta, GA
Veterinary Assistant December 2017 - August 2019
- Assisted with restraining animals for examination, administered fluids, clipped nails, expressed anal glands, and gave baths, fed and walked boarding animals after regular business hours

Research

The Pennsylvania State University

Staniar Lab August 2021 - present
Undergraduate Research Assistant
- Assist Dr. Staniar and graduate student with collecting fecal samples, drawing blood, handling yearling horses, analyzing heart rate data, measuring fecal pH, and assisted veterinarians with gastroscopies
- Conducted an honors thesis evaluating equine fecal pH measurement methods and storage protocols

Gould Lab March 2023 - present
Undergraduate Research Assistant
- Neurobiological Investigation of Learning and Memory (NILA) research group
- Assist with nicotine implantation surgeries, brain slice preparation, Golgi staining, and Sholl analysis

Leadership Activities

- Undergraduate Teaching Intern (UGTI), *Penn State Pharmacology Class 451* 2023
- THON Merchandise Chair, *Penn State Equestrian Team* 2020 - 2022
- Elected THON Dancer, *Penn State Equestrian Team* 2022
- Donor Alumni Relations Chair, *Penn State Equestrian Team* 2022 - present
- Secretary, *Equine Research Team at Penn State* 2022 - present

Relevant Coursework

Pharmacology • Neurobiology • Functional Neuroscience • Physiological Psychology • Animal Nutrition