IMMEDIATE EFFECTS OF GRAPE POMACE ON HORSE (*EQUUS CABALLUS*) FECAL MICROBIOTA AND PH

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SIGNATURE PAGE

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ABSTRACT

The demand for wine has led to an increase in its by-product, grape pomace. Constant manufacturing of wine has led to 8.2 million tons of grape pomace produced worldwide. Utilizing some of this pomace as animal feed has the potential to reduce wine production waste and provide a new nutritive feed to animals in agriculture. This study observes the fecal pH, *Lactobacillus* spp., methanogen, and total bacteria population of horses from the Kellogg Arabian Horse Center that were supplemented with a low dose of dried grape pomace (DGP). The hypothesis for this study was that there will be change in fecal parameters due to the supplementation of polyphenols and dietary fibers from the pomace. Seven horses were fed a control and treatment diet for 21 days in a crossover study. Treatment diet consisted of supplementation of 100 milligrams of DGP per kilogram of horse's body weight (mg DGP/ kg BW). Fecal pH range for this study was between 7.39 – 7.77 with no significance difference between control and treatment group ($P = 0.75$). Overall, there was no significant difference found in fecal populations of total bacteria ($P = 0.95$), *Lactobacillus* spp. ($P = 0.33$), and methanogens ($P = 0.61$). In conclusion, horses in this study fed 100 mg DGP/kg BW showed no significant changes to fecal pH and microbes observed. As research is limited for grape pomace as horse feed, further investigation is needed in other physiological parameters, such as polyphenol metabolism, but there seems no immediate effects or concerns when feeding a low dose of DGP.

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CHAPTER 1: INTRODUCTION

Wine is one of the most prominent drinks around the world known originally for its affiliation with religion. Possibly starting with the discovery of grapes from the Vikings, centuries later it has become one of the most widely consumed alcoholic drinks in the world, constituting 40% of alcohol consumed, alongside beer (another 40%), in countries like Canada, the United Kingdom, Australia, and the United States from 1960-1985 (Pinney, 2007; Spawton, 1990). Unfortunately, due to rising popularity, the number of wineries have increased, leading to excess waste production. A by-product (waste) formulated from winemaking is grape pomace and it can build up to millions of tons.

Recently grape pomace has been incorporated into livestock's diet as a waste-control measure to neutralize by-products produced by wine manufacturing. To continue with the trend, this study plans to incorporate wine pomace into horses' diet. Grape pomace is known to be prolific in polyphenols compared to other by-products and the leading factor in considering the residue as a feed (Chedea et al., 2016; Deng, Penner, & Zhao, 2011). The concern with feeding polyphenols is the antimicrobial and antioxidant properties that can affect the microbial community inside a horse's cecum. Horses are hindgut fermenters meaning a large portion of digestion occurs by bacterial fermentation in the large intestine in a chamber called the cecum, where cellulose-based compounds are converted to volatile fatty acids by the microbes and provide digestible energy to the animals. Fortunately, as hindgut fermenters, horses still have other digestive processes in the foregut where other nutrients such as proteins, fats, and polyphenols can be digested and absorbed in the small intestine.

To gain a greater understanding of the effects of grape pomace on horses, further research needs to focus on a variety of physiological parameters. Understanding polyphenol (nutrient of interest) biochemistry and how it is metabolized in the horse gastrointestinal tract determines if grape pomace would be a valuable feed in the horse industry. Continuing with research focusing on fecal microbiota and pH gives an insight of the microbial community that is functioning within the cecum of the horse. Maintaining the cecal microbiota is vital to horse nutrition and studying fecal matter is a financially feasible approach to understanding cecal status. The high fibrous dry matter of grape pomace is more than capable of reaching the fermentation site of horses and studying the changes it has on feces is a contribution to understanding the potential of grape pomace as feed (Kolláthová et al., 2020). Wine production and the by-products formed are a growing concern due to the waste yielded and utilizing wine by-products as feed for horses (and other animals) can assist in reducing wine production waste while introducing an alternative feed to the equine industry.

CHAPTER 2: LITERATURE REVIEW

One of the most prominent and historic alcoholic drinks in the world has caused major globalization of an industry and yet there has been a recondite ramification not known to the public. Wine is the leading global alcohol, right behind beer, and its mass production to meet consumer demand has consequently produced a plentiful amount of waste associated with wine manufacturing. A proposed resolution to assist in waste reduction from wine production includes feeding the excess by-products to animals as most goes to landfills or left to rot. The primary by-product formed during wine production is called grape pomace and it consists of grape pulp, skin, and seeds. Still full of rich nutritive content, such as polyphenols and dietary fibers, grape pomace has plenty of potential benefits to provide to animals. One of these animals that can benefit from grape pomace are horses, and with an immense market worldwide, a grape pomace feed product can surely contribute to the wine industry's waste management. Previous research will dictate the capability of utilizing grape pomace as a feed in the horse industry. Past studies involving animals and grape residue, fruit by-products, or feeding trials contribute to forming a logical experiment to analyze the effects of grape pomace on horse's fecal microbiota and pH.

Wine Productions and Its Effects

Wine production is a multibillion-dollar industry that first started in Europe and eventually globalized affecting countries such as the United States, Argentina, South Africa, Chile, and Australia. Since 2004, the United States is responsible for producing 2.4 billion liters of wine, composing 8% of the world's production (Hussain, Cholette, & Castaldi, 2008). Twelve years later, United States wine production increased by 29.2%

producing approximately 3.1 billion liters. California is the most concentrated area of winery locations in the United States with 84.3% (2.6 billion liters) of wine production occurring in the state since 2016 (Alston et al., 2018). Due to adequate seasonal weather, California has become one of the leading growers of grapes (and general agriculture) leading to the rise of wine production since the late 19th century.

Grape Pomace

Winemaking consists of extracting juice from grapes that will later be fermented, leaving a variety of by-products. One of these common by-products yielded in the wine industry (and grape juice production) is grape pomace, which consist primarily of stems, skins, pulp, and seeds; different variations exist such as seedless pomace and grape seed extract (Beres et al., 2017). With wineries and juice companies extracting as much fluids from the fruit as possible, organic residue (grape pomace) that has been deteriorated is left, constituting an estimated 20-30% of the total weight of grapes processed (Dwyer, Hosseinian, & Rod, 2014). With 54 billion kilograms of grapes processed each year worldwide, 8.2 billion kilograms of grape pomace is generated as well, raising a concern for waste management (Arvanitoyannis & Varzakas, 2008).

The composition of grape pomace continues to be a great source of various nutritive properties. For example, grape seeds from pomace are utilized to produce grape seed oil known for its richness in unsaturated fatty acids (Bail, Stuebiger, Krist, Unterweger, & Buchbauer, 2008). Due to its rich source of sugars and low proportions of pectin, grape pomace has also become a great source for a high dietary fiber ingredient (Valiente, Arrigoni, Esteban, & Amado, 1995). Valiente et al. (1995) proposed that the insoluble fibers found in grape pomace can support bowel regulation and water retention in humans. The more notable composites found in grape pomace (and wine) are phenolic compounds; a class of these compounds are polyphenols known well for their antioxidant properties (Chedea et al., 2016). Combining these three aspects of rich fatty acids, fiber, and phenolic compounds make grape pomace a great resource for multiple products and feed for animals.

Properties of Grape Pomace

Grape pomace has been a contentious topic regarding its use as animal feed due to its contingency of high sugar content in grapes. Animals with anaerobic microbe populations in their digestive tracts (e.g., cattle, sheep, goats, and horses) are accustomed to low carb and starch diets. Feeds high in sugars, starches, and other carbohydrates tend to cause high lactate production in the rumen and/or cecum thus causing health problems in the animal (Owens, Secrist, Hill, & Gill, 1998). California being one of the leading grape producers in the United States, wine grapes have substantially changed from their teleological origins due to climate control and advanced viticulture practices (Alston et al., 2018). With California's tempered weather and resources, sugar content in wine grapes have considerably increased over the past three decades. Starting in 1980, the average sugar composition of wine grapes (red and white) in California has increased by 9% since 2008; that's a jump from 21.4 degrees Brix (1980) to 23.3 degrees Brix (Alston, Fuller, Lapsley, & Soleas, 2011). Glucose and fructose are the predominant sugars found in grapes and concentrations can vary from 45.86 to 131.04 mg mL^{-1}, with wine grapes having more sugars and acids than table grapes (Liu et al., 2006). Understanding grape nutritive content is vital to understand grape pomace composition to prevent conditions like acidosis in fermenters like cattle and equine.

Polyphenols

Polyphenols are micronutrients (a type of phenolic compound) that have gained high interest for their importance in physiological function. Phenolic compounds are one of the most distributed natural products in the plant kingdom, bearing an aromatic ring with one or more hydroxyl substituents in their structure (Beres et al., 2017). Flavonoids, stilbenes, and tannins are all polyphenols found in grape pomace containing a rich source of antioxidant agents and, in general, phenolic antioxidants have shown to be vital for physiological metabolism and chemistry (Hathway, 1966; Rockenbach et al., 2011). Research has demonstrated slight health benefits in animals fed a high polyphenol diet including improvement in intracellular metabolism (Manach et al., 2004). Polyphenols are the most abundant compound found in grape pomace and constitute a significant role in determining the impact on equine metabolism. A previous study has revealed supplying polyphenols via grape pomace show high retention rate of polyphenols in ruminal fluid, but observations of limited radical dilapidation indicate there is low antioxidant activity (Chedea et al., 2016). With antioxidant activity levels rather low in the ruminal fluid, the potential to utilize grape pomace in animals with large portions of microbial fluid increases significantly, since some evidence is shown that microbial populations can withstand constituents of grape pomace. Gessner et al. (2017) provide concise evidence in their review article about polyphenol properties that plant polyphenols have the potential to combat oxidative stress and provide anti-inflammatory relief on farm animals, but side effects include impairment of nutrient digestibility.

Polyphenols are notorious for their association with antioxidant properties. Hathway (1966), author of “Metabolic Fate in Animals of Hindered Phenolic

Antioxidants in Relation to their Safety Evaluation and Antioxidant Function," defines food antioxidants as a means to preserve the nutritional value of the diet of animals and man. Antioxidants inhibit deteriorative reactions during metabolism and understanding metabolic pathways of animal species is vital to understand physiological usage in animals to increase performance and desired qualities. Feeding polyphenol rich feedstuff, such as grape pomace, provides antioxidants to support certain physiological functions, but further research is required to demonstrate copious amounts don't hinder microbial fermentation in foregut and hindgut fermenters.

Flavonoids

Flavonoids are a secondary and popular class of polyphenols that are responsible for favorable biochemical effects in mammalian physiology. Comprised of subcategory compounds like flavanols, flavonols, and anthocyanins, flavonoids provide some health-promoting effects (as previously mentioned) including anti-inflammatory relief, antioxidative components, anti-mutagenic properties, and the capacity to modulate enzymatic function (Panche, Diwan, & Chandra, 2016). Bioactivity of these phenolic compounds are fairly low in mammals due to their minimal concentrations, but supplementing diets rich in fruits and vegetables introduces flavonoid content supporting cellular metabolism (Passamonti et al., 2009). Within polyphenols, dietary flavonoids represent an essential source of antioxidants, but flavonoids also have antimicrobial effects that may not situate well the microbial fermentation that occurs in horses (Pietta, 2000).

Flavonoids as antimicrobial constituents in feeds have the potential to provide health benefits, but also alter highly efficient microbiomes in animals with fermentation capabilities. Flavonoids antimicrobial properties are believed to originate in plants to produce colors in flowers and act as a mechanism to prevent infection from pathological fungi on leaves (Harborne & Baxter, 1999). With antimicrobial activity a key component of polyphenols, it has been a highly researched topic as a healing source for diseases and infections (Cushnie & Lamb, 2005). Cushnie and Lamb further elaborate on their review about flavonoid antimicrobial activity that previous reports of flavonoid extract from *Hypericum*, *Capsella*, and *Chromolaena* demonstrated antibacterial activity in remedy usage. Propolis, a resin-like material produced by bees, is also a flavonoid-rich compound the has attested to subdue periodontopathic bacteria, like *Escherichia coli* and *Staphylococcus aureus* (Bankova, Popov, & Marekov, 1983; Gebara, Lima, & Mayer, 2002). Using flavonoids to combat bacterial infections has done wonders in the medical field, as evident in a preliminary retrospective study depicting flavonoids as an adjunctive treatment for equine lymphoedema (Katrinaki & Diakakis, 2015). Additional research also suggests flavonoids contain antifungal and antiviral capabilities, with inhibitory effects on viral enzymes (Bylka, Matlawska, & Pilewski, 2004). However, introducing such a phenomenon may have side effects on gastrointestinal tracts that have a diverse micro ecosystem, like in horses, cattle, and sheep.

Flavonoids have the potential to dispatch certain bacteria, seen as positive health remarks, but further analysis is required to make certain that polyphenol quantities in feedstuff (e.g., grape pomace) don’t have negative effects on the microbiome of animals. Antagonistic outcomes from excess flavonoids include deterioration of bacterial populations essential to animal gut health. In horses, *Streptococcus bovis*, *Streptococcus equinus*, and strains of *Lactobacillus* are prominent bacterium in the equine gastrointestinal tract that produce copious amounts of volatile fatty acids (VFAs) that are essential to horse nutrition. (Al Jassim et al., 2005; Daly et al., 2001). An ample amount of flavonoid concentrations in fermentation sites of animals can suppress microbes from functioning properly and synthesizing fatty acids necessary for physiological maintenance (Li & Tian, 2004). Although flavonoids are encouraged to be incorporated in a horse's diet for their antioxidant properties, antimicrobial activity raises caution in feeding guidelines, but if done correctly antimicrobial properties can be utilized as an advantage.

Properly portioned flavonoid-rich feedstuff may use antimicrobial activity for health progress or treat ailments in large animals with fermentation capabilities. Bacterial colonies like *Streptococcus* and *Lactobacillus* convert carbohydrates from feed into VFAs and lactic acid, resulting in an acidic environment of the horses' cecum (Argenzio, Southworth, & Stevens, 1974). Flavonoids from grape seed extractions have the potential to inhibit growth and diminish viability of certain lactic acid producing bacteria (e.g. *Oenococcus oeni* and *Lactobacillus hilgardii*) (Figueiredo, Campos, de Freitas, Hogg, & Couto, 2008). Thus, exploiting wine residue to reduce some of these lactic acid producing bacteria in horses benefits gut health. During hindgut fermentation in horses, feed that is easily fermentable produces a profuse amount of lactic acid eventually making the cecum in an undesirable acidic condition, leading to disorders such as acidosis, laminitis and other equine metabolic syndromes. Flavonoids antimicrobial activity can also be advantageous in being able to remove or suppress pathogenic bacteria via its phytochemicals (Kamboh et al., 2015). Harnessing flavonoids to pacify the equine cecal microflora and relief/prevent equine metabolic disorders increases interest to introduce more polyphenol-rich feed into the horse industry.

Tannins

Tannins are another class of polyphenols that are similar to flavonoids and have extensive research with animal nutrition. Tannins are categorized as hydrolyzable tannins and proanthocyanidins; with the former perhaps the most ubiquitous off all polyphenols (also known as condensed tannins). Condensed tannins originate from flavanol units and are polymers of said flavonoid group, but contain more substituent groups than flavonoids increasing antioxidant, antimicrobial, and antimutagenic activity (Schofield, Mbugua, & Pell, 2001). Due to higher metabolic reactivity, tannins had astigmatisms of toxic traits in animals until proper research garnered substantial evidence to clarify the value of tannins. Mueller-Harvey (2006) articulately elucidates the positive and negatives of condensed tannins in his review article "Unravelling the conundrum of tannins in animal nutrition and health" as a debate arose regarding the feeding of tannin compounds to production animals. Similar to flavonoids, managed intake of tannins in animals provide physiological benefits and excess amounts will become toxic.

Tannins are capable of providing beneficial nutritional effects to animals in appropriate amounts. Mueller-Harvey states ruminants with condensed tannins in their diet improves utilization of dietary proteins, growth rates of liveweight and wool, milk yield, fertility, immunity, and welfare. The array of benefits tannins contribute to animal physiology are possible due to their chemical structure that allows them to bind to proteins. This characteristic primarily pertains to ruminants as tannins can bind to proteins, forming tannin-protein complexes, and bypassing them through ruminal digestions and making proteins readily available for small intestine digestion (Patra & Saxena, 2011). Horses' microbial fermentation occurs after the foregut, but little research limits to what is known on how tannins (and other polyphenols) are processed in hindgut fermenters like equines.

Extensive research on ruminants is anticipated for their contributions to food products and compounds that can support production will sufficiently be studied assuring safety of animals and maximizing product yield (e.g., meat, milk, wool, etc.). Tannins cause concern because, unlike flavonoids, there are little identifiable bacteria known to catabolize tannin compounds in microbial digestion (Tabasco et al., 2011). Conflicting reports depicted extreme tannin consumption can reduce animal productivity by reducing voluntary feed intake and digestibility of nutrient and mineral absorption (Kumar & Vaithiyanathan, 1990). Prior research indicates ruminants possess mechanisms to overcome tannin rich feeds, including enzymes in their saliva and rumen (Makkar, 2003). Many researchers believed the adaptation was a defense mechanism against tannins, and like flavonoids, antimicrobial properties make them potent to depreciate the microorganism ecosystem, raising concerns of incorporating tannin-rich feedstuff into animals' diets.

Tannin interactions with microbial communities have heavily been observed to further clarify the antimicrobial activity polyphenols are known for. Albeit mechanisms are not fully discovered, tannins inhibit enzyme and substrate interaction with microorganisms in ruminal fermentation and reducing microbe productivity (eventually dying), supporting claims stated earlier with condensed tannins preventing proteins from undergoing microbial digestion (McSweeney, Palmer, McNeill, & Krause, 2001). When condensed tannins interact with proteins, forming tannin-protein complexes, dietary proteins are no longer readily available for those microbes that need those macronutrients and causes microbe degradation (e.g. *Streptococcus bovis)* which are integral to herbivore digestion (Bhat, Singh, & Sharma, 1998; Osawa & Sly, 1992). Yet, animals have evolved to withstand high concentrations of tannins whether it be enzymes in their saliva to neutralize polyphenols, microbes adapting to tolerate tannins, or introducing microflora that are capable of degrading tannins (Scalbert, 1991).

Dietary Fibers

Cereal grains are a common feed in the horse industry to provide high energy intake for racing, exercising, and maintenance, but have been associated with pathological issues such as stomach ulcers, acidosis, laminitis, and colic (Costa et al., 2012; Rowe, Lees, & Pethick, 1994; Swyers et al., 2008). Beet pulp has been used as a substitute for a high-starch diet, as the high-fiber feed provides adequate energy to maintain weight and to perform equally as well as the horses on the high-starch diet (Crandell, Pagan, Harris, & Duren, 1999). Utilizing sugar beet pulp as a model for grape pomace will assist in determining grape pomace utilization since both are by-products from the agriculture industry and have elevated dietary fiber content (Kelly, 1983; Yu & Ahmedna, 2013).

An *in vitro* fermentation study conducted by Murray, Longland, and Moore-Colyer (2006) inoculated horse feces and observed the reaction when a mixture of ground dried lucerne (e.g. early bloom alfalfa) and beet pulp rations were added. Six different lucerne: beet pulp combinations were tested; 100:0 (pure lucerne), 80:20, 60:40, 40:60, 20:80, and 0:100 (pure beet pulp). Results included a quadratic response to pH of culture fluid and linear response to total volatile fatty acid concentration. As beet pulp gradually substituted lucerne, pH of culture fluid became more alkaline until beet pulp was greater in volume (40:60, 20:80, and 0:100) then pH decreased. Culture pH formed a quadratic pattern from 6.72 (pure lucerne), peaking at 6.81 (60:40) and decreasing to 6.61 (pure beet pulp). Compared to total volatile fatty acid concentration, a linear reaction transpired as concentration mixtures gradually increased with beet pulp levels. Starting at 54.2 millimoles per liter (mmol/l) with pure lucerne and eventually reaching 72.4 mmol/l for fermentation capsules fed pure beet pulp. P. Kelly, author of *Sugar Beet Pulp – A Review*, justifies the large fiber content of beet pulp is not all digestible in the horse gastrointestinal tract. Some of the feedstuff will be fermented in the cecum but some portions are insoluble, perhaps elucidating when greater portions of beet pulp are available more soluble dietary fibers are readily available as well resulting in microbial fermentation to excel corresponding with an acidic environment and greater volatile fatty acid production. Grape pomace is also a high-fiber compound, and a similar conclusion could be assumed, disregarding the phenolic compounds.

Total dietary fiber of grape pomace varies within the grape cultivar and impacts the digestibility in the horse gastrointestinal tract. Via an *in vitro* indigestible fraction, indigestible dietary fibers of red and white grape pomace were calculated. Bravo and Saura-Calixto (1998) determined roughly 60% of dry matter grape pomace is indigestible (*in vitro*) and a majority is due to insoluble dietary fibers. Insoluble fibers are associated with bowel regulations and water retention in humans, but superfluous amounts can disrupt the microbial ecosystems found in fermenters (Valiente et al., 1995). Cabernet sauvignon is the most popular wine grape around the world and an estimated 53% dry matter of grape pomace skins is composed of total dietary fiber (Bowers & Meredith, 1997; Deng et al., 2011). Deng et al, goes further into detail, specifying less than 1% of dietary fibers are soluble and the rest are insoluble. Other red wine grape cultivars displayed similar characteristics with approximately 50% of total dietary fibers constituting as insoluble fibers in Merlot and Pinot Noir grape pomace skins. Compared to white grape varieties (e.g., Muller Thurgau and Morio Muscat), total dietary fibers were less than 30% with a majority of dry matter composition relegated to soluble sugars. Important to note is dietary fiber is defined as plant residue comprised of plant cell walls that is resistant to hydrolysis in human metabolism (Howlett et al., 2010; Trowell, 1976). The term is catered to human metabolism, but the wide margin of insoluble dietary fibers found in grape pomace still deliberates valuable knowledge needed to understand grape pomace digestibility in horses.

The Gut Microbiome and Equine Gastrointestinal Tract

The gut microbiome (i.e., microflora) is found in nearly all mammals classifying them as metagenomic, and evolution has adapted the flora into different functions (Ley et al., 2008). Some functions of the gut flora include immune capabilities, where intestinal microbes will digest and eliminate pathogens (O'Hara & Shanahan, 2006). Most importantly, the gastrointestinal microorganisms have a symbiotic relationship within mammals providing additional digestion support of compounds (like fibrous plants) that are not capable of being broken down by physiologic digestive enzymes. Omnivores and carnivores have a fat and protein based diet, thus limiting microbial population but microbes still participate in pathological and compound digestion (Bauer, Williams, Smidt, Mosenthin, & Verstegen, 2006). Herbivores, which comprise approximately 80%

of mammals, contain microbes that digest and convert abundant sources of grasses and roughages that don’t contain many nutritive properties into valuable sources of energy (Stevens & Hume, 2004). In the horse and agriculture industry, it is important to understand animal digestion patterns, especially in herbivores, to maximize growth and performance in an efficient time.

The horse digestive system is an aberrant unit as it consists of a simple stomach and cecum with fermentation capacity. Due to these functions, horses are classified as hindgut fermenters, where they use metabolites in their foregut (stomach) to breakdown feed and then nutrients, such as fats, carbs, and proteins, from the feed are absorbed in the small intestine (Moore, Melton, Carter, Wright, & Smith, 2001). Lignin and other fibrous compounds are digested by anaerobic microbes in the cecum that produce volatile fatty acids as waste but are essential to equine (and ruminants), where they are absorbed in the large intestine alongside water (Argenzio et al., 1974; Owens et al., 1998). When horses first consume their feed, usually forage, the passage rate through the stomach and small intestine is relatively quickly compared to the hind gut. As feed takes an average of five hours to pass through the foregut, it takes approximately 35 hours to pass the hindgut and this is due to feed being retained in the cecum for microbial digestion (Van Weyenberg, Sales, & Janssens, 2006). The largest amount of microflora is found in the cecum, where a symbiotic relationship results in microbes being fed and volatile fatty acids produced by those microbes that provide a significant energy source to horses and ruminants. Diet composition greatly impacts the hindgut microbiome as production of volatile fatty acids vary within feed composition (forage versus concentrates), thus being vital to equines for owners to understand feeding management (Julliand & Grimm, 2017).

When horses defecate, microbe populations are released which can be observed to view gut health of a horse. Horses do not tend to have a fully diverse microbiome until they are about two months old, because foals are born with a very limited microbial population inherited by their mother during parturition; coprophagy and sampling of their surroundings introduces new microbes into their cecum microbiome (De La Torre et al., 2019). Using feces is an efficient method towards studying microbiota because contact with the animal is reduced, preventing stress; fraught with ethical concerns, previous procedures included fistulation, anesthetics, and euthanasia (Julliand & Grimm, 2016). Unfortunately, fecal microbiota best summarizes microbial data from the dorsal and small colon, not directly from the cecum, although pre existing findings demonstrate there is still ample information to gain from fecal analysis in hindgut fermenters (Dougal et al., 2012; Ericsson et al., 2016).

The gut microbiome of herbivores is a diverse community of microorganisms essential to horses, as well as ruminants and other animals with fermentation capabilities (e.g. rabbits, rhinoceros, tapirs). Microbes that live within the cecum (site of fermentation) of horses consist generally of bacterium, protozoa, fungi, and archaea (Daly et al., 2001; Julliand & Grimm, 2016). A collection of past studies suggest the bacterial phyla, *Firmicutes,* is dominant in the microbial community of the equine gut, followed by *Bacteroidetes* (Dougal et al., 2012; Zhao et al., 2015). Contradictory, breed, age, health and diet can affect microbial community with previous research collected by E. Venable, Bland, McPherson, and Francis (2016) depicting *Proteobacteria* and *Verrucomicrobia* as other possible dominant phyla; yet, studies that used fecal samples for microbial analysis resulted in *Firmicutes* being the dominant phyla regardless of age, breed, and diet (Costa et al., 2012; Dougal et al., 2014; Morrison et al., 2018).

More detailed analysis shows a diverse bacterial community in horse feces. *Lactobacilli*, *Streptococci*, and *Bifidobacterium* species are highly prolific in horse feces (Endo et al., 2007). Other microbes found in horse feces have functional purpose in the cecum such as cellulolytics and amylolytics. *Lactobacillus* spp. is a bacterium that is detected virtually in every horse and is the predominant species in feces regardless of breed, forage, or environmental factors (Endo et al., 2009). Diets and diet changes are the primary cause of differential change on fecal microbiota. Dietary starches (e.g., concentrated feeds) effectively change cellulolytic, lactobacilli, gram-positive cocci, lactate-utilizers and amylolytic populations in the fecal microbiota (Harlow et al., 2016). When conducting research with fecal matter and microbiota, understanding and standardizing the local fecal microbiome is crucial for proper results.

Feed and Pomace Digestion in Horses

The digestive passage of horses consists of many physiological mechanisms, in which grape pomace will be digested in different forms. The mouth is where mechanical and chemical breakdown of any feed begins. Horse saliva is virtually water and contains very minimal digestive enzymes, limiting its function as lubricant for ingested feed and a buffer for optimal bacterial digestion in the stomach (Varloud, 2006). The digesta travels down the esophagus and into the stomach, where hydrochloric acid is secreted by parietal cells. In the stomach, digesta is broken down via secretions released by a variety of cells in mucosa layers of the stomach. In horses, the fundus area ($6< \text{pH} < 7$) is less acidic than the pyloric region ($\text{pH} < 4$) of the stomach and bacterial fermentation can occur in the fundus resulting in minimal bacterial digestion of polyphenols and dietary fibers (Merritt & Julliand, 2013). Much of the digesta is degraded by stomach secretions and ready to pass through the small intestine in the form of chyme for nutrient digestion. Chyme is acidic since it came from the stomach thus an assortment of pancreatic juices are released in the small intestine (jejunum/ duodenum) to neutralize the chyme for efficient digestion. Bile salts and lipase are released by the biliary system and pancreas, respectively, to assist in fat degradation. Amylase is another enzyme released by the pancreas and found abundantly more in horses fed high forage diets (Roberts et al., 1974). Amylase, specifically known as α-amylase, hydrolysis starches and converts them into sugars for proper digestion in the small intestine.

The small intestine is where polyphenol metabolism from the dried grape pomace and other feeds will continue. The metabolism of polyphenols is limited in research and is a topic that still needs further exploration. An estimated 5 - 10% of total polyphenol intake is believed to be absorbed in the small intestine of monogastric animals (Chiva-Blanch and Visioli, 2012). Brenes et al. (2015) and Lipinski et al. (2016) elaborate about polyphenol metabolism on their review of polyphenols from grape by-products in monogastric nutrition. The complex structures of polyphenols are what determine the metabolism rate in the gastrointestinal tract. Polyphenols with less complex structures, such as flavones and flavanols, are absorbed by the enterocytes and are prone to hydrolysis and biotransformation, where the end products travel to the liver and then the circulatory system. The rest of the of the polyphenols travel to the large intestine where intestinal bacterial enzymes from the lumen further decompose polyphenols into absorbable phenolic metabolites. Bioactive use of the polyphenols in the body are believed to be derived from these phenolic metabolites. Excess polyphenols and metabolites are recirculated through bile for reabsorption or are excreted through urine.

Plant components that were not digested in the small intestine make there way into the large intestine (colon) and the cecum. Dietary fibers from grape pomace and forages enter the cecum where fibrolytic activity is high. Bacteria and fungi attach to fiber particles and release enzymes to initiate cellulolysis. The monomeric sugars are then hydrolyzed in bacteria resulting in pyruvate and eventually volatile fatty acids (VFAs) and gases (Merritt and Juliand, 2013). The main VFAs produced in the colon are acetate (74.8%), propionate (17%), and butyrate (6.2%) (de Fombelle et al., 2003).

Changes in Fermentation Characteristics

Analysis of fecal pH gives further perspective of the gut microbiome and how the microorganism ecosystem was altered from diet changes. A review proposed by Julliand and Grimm (2016) stated the microbiome of the horse hindgut is a complex arrangement of anaerobic and aerobic microorganisms undergoing fermentation and feces reflects the condition in which the hindgut microbiome is in. Naturally, the site of fermentation (the cecum) is acidic due to lactic acid constantly produced from the microbes and how acidic the cecum becomes varies with the animal's diet (Al Jassim et al., 2005; Argenzio et al., 1974). Teleological feeding behavior of horses is best represented as grazers, more specifically browsers as they spend a majority of their daytime foraging in grass fields. High-fiber feeds, consisting of soluble and fibrous carbohydrates, are what microbes are accustomed to, producing volatile fatty acids to be used by the horse as a result and minimizing lactic acid production, until high-starch compounds are introduced (Fonnesbeck, 1968).

Although horses were evolved to graze for hours in a single day, domestication has restricted natural feeding behavior and adapted them to alternative feeds to meet physiological requirements. Herbivores evolved to get access to complex carbohydrates found in plants, such as celluloses and resistant starches, to accommodate for bacterial fermentation and in return specific digestive organs enlarged, like in the horse and its hindgut (cecum) (Ley et al., 2008). Cell wall carbohydrates, such as cellulose and pectin, prevent humans from fully digesting a wide assortment of plants, but the diverse microbial community found in horses and other herbivores feed on those carbohydrates allowing plants to be their host's main food source. Even with the capability to ingest plants, bacterial fermentation is a long process due to the decomposition of cell wall carbohydrates with the mean retention time of digesta in the cecum of horses being 35 hours (Julliand & Grimm, 2017; Van Weyenberg, Sales, & Janssens, 2006). Fibrous plants undergo hours of microbial fermentation inside horses, but as the industry evolved to house horses in stalls so did their diet resulting in further research to understand the changes undergoing in the microbiome.

Horses housed in stalls have their grazing behavior removed and instead replaced with portioned meals distributed throughout the day. Horses need to be fed a mixture of forage and concentrates to meet nutritional requirements equivalent to what they would receive grazing in grass fields all day. Hays and grains fed to horses consist of high starches that act as a nutrient supplement, but unregulated feeding causes disruption to the microbiome. Julliand et al. (2001) depicted the microbial profile of ponies as they were fed three dietary hays: barley ratios. A diet with 50% hay and 50% barley led to a higher concentration of total bacteria compared to a diet of pure (100%) hay. Compared to forages, concentrates such as grains and cereals are easily fermentable due to their lack of cell wall compounds. Another study observing microbial population in ponies showed the same overall results; after different hay: concentrate ratio were fed to ponies, bacterial concentrations were higher in diets composed of 60% alfalfa and 40% concentrate compared to a diet of 90% alfalfa and 10% concentrates (Moore & Dehority, 1993). Introducing other feeds besides forages changes the microbial concentration, which proposes the limits in which feeds outside grasses and hays can be introduced and become a staple for a horse's diet, such as grape pomace. Observing immediate changes in the fecal aspect of horses can theorize changes in the gut microbiome.

Abrupt dietary changes are common in the horse industry and knowing when and how to introduce a new feed is vital for horses' health. Microbial populations and fecal pH suddenly spike if horses are transitioned from pastures to concentrate feeding (van den Berg, Hoskin, Rogers, & Grinberg, 2013). Assertions made by van den Berg et al. support the argument that when transitioning diets in horses they should be done gradually to avoid metabolic and digestive disorders. In the study, horses were transitioned over from pastures to a concentrate-based diet and fecal pH rose in the initial days from ~6.2 on Day 0 to ~6.6 by Day 3 of introduction of concentrates. After Day 3, fecal pH decreased to return to its initial pH of ~6.2, but when horses were abruptly transitioned back to pastures fecal pH increased again capping off at ~6.45. Results from the fecal pH correspond with the specific bacterial population recorded in which *Streptococcus* spp. and *Lactobacillus* spp. flourished in the preceding 13 days of the concentrate diet and decreased as the diet transitioned to pasture grass. Results support Juliand et al. (2001) findings in which Lactobacilli and Streptococci significantly increased with added concentrates, and overall, with Moore et al. evidence, affirm bacterial concentrations increase as concentrates are added to an equine's diet.

A study similar to van den Berg et al. was conducted in New Zealand observing fecal pH of horses maintained on pasture while supplemented with grain (Rogers, Eastwood, Gee, & Firth, 2004). Twelve horses were divided evenly into two treatment groups and kept under identical pasture management. The control group had grass available ad lib, while the grain supplementation group was provided with 6.16 kg of oat grain per horse per day. Just like van den Berg at al. outcome, Rogers et al. states they were unable to demonstrate a decline in fecal pH when soluble carbohydrates (crushed oats) were introduced into horses' diet, contradicting previous research of concentrates of high soluble carbohydrates reducing fecal pH (Rowe et al., 1994). Reviewing the equine digestive system, some soluble carbohydrate absorption still occurs in the small intestine, with fibrous compounds and carbohydrates reaching the cecum A possibility was oat grain was digested in the stomach and carbohydrates were absorbed in the small intestine, limiting a small amount of grain to reach the cecum (Moore et al., 2001). Another plausible reasoning explained by Rogers et al. was since horses had unlimited access to pasture grass, all the forage they consumed negated the effects of the ingested crushed oats. With a high forage: concentrate ratio, the microbe ecosystem, composed primarily of anaerobic bacteria, are acclimated to consume fibrous compounds that are slowly fermentable (Santos, Rodrigues, Bessa, Ferreira, & Martin-Rosset, 2011).

A research project in North Carolina adds another perspective to pasture feeding with diet change and fecal pH (Glunk, Pratt-Phillips, & Siciliano, 2013). Horses' dry matter intake rate, dietary energy intake, and fecal pH were measured when groups were placed in pastures for three, six, nine, and twenty-four hours; the three- and six-hour pasture groups were offered grass hay. What followed was average fecal pH was highest in pasture groups of 24-hours (7.67) and decreased as horses had less access to pastures; pH of 7.21, 7.21, 7.34 for three, six, and nine-hour pasture access, respectively. Noticeable aspects about this study is fecal pH remained alkaline (pH>7) compared to previously mentioned studies where they were all acidic (pH<7), but differences are bound to occur due to dietary differences and management practices (Cohen, Gibbs, & Woods, 1999; Venable et al., 2017). Contrary to prior experiments, no concentrate source was provided and yet there was change in fecal pH, demonstrating changes can occur in the microbiome with different forages. Feed quality dictates retention time during digestion, and depending on the grass hay fed, fiber digestibility seems to have been higher with horses provided the grass hay due to limited duration in pastures resulting in a lower fecal pH compared to group that had no hay provided to them (9- and 24-hour group) (Miyaji, Ueda, Hata, & Kondo, 2011).

An *in vitro* study analyzing fermentability of various fruit pomace was conducted in Japan utilizing microbes found in the horse fecal matter (Hwang et al., 2017). Residual by-products from freshly squeezed apple, carrot, grape, and citrus were fermented under individual solid-state conditions containing 60% soybean meal, 40% fruit pomace, and 60% moisture content. Strains inoculated from horse feces were *Lactobacillus, Weissella,* and *Bacillus* and grown during fermentation to be isolated and collected, alongside pH and lactic acid concentration. Firstly, pH dropped with the presence of fruit pomace within 12 hours of fermentation. All fruit groups depicted drop in pH as initial pH ranged from 5.45-6.25 and became even more acidic after a 12-hour period, resulting in pH of

4.52-5.01. After a 24-hour period from initial recording, final pH dropped even more; 4.47-4.66 pH. Corresponding to pH decline, lactic acid concentration rose with the addition of pomace. Sugars and starches found in the fruit support growth of certain bacteria, thus promoting access production of lactic acid, which causes an acidic environment (Tabasco et al., 2011). This study analyzes the changes in a simulated environment (in vitro) and does not replicate the digestive unit of a horse, where their primary feed would consist of a forage and not soybean meal. Albeit, there is significant drop in acidity inside the fermentation capsules, a forage mixture would have needed to replace the soybean meal to better depict fermentation behavior in a horse and proof if acidity would have severely dropped.

Grape Pomace as Animal Feed

Research regarding grape pomace as animal feed has been limited, as 3% of grape pomace waste is used as animal feed, but the advent of studies have shown great progression for utilization of grape pomace in the livestock industry (Dwyer et al., 2014; Kalli, Lappa, Bouchagier, Tarantilis, & Skotti, 2018). Brenes, Viveros, Chamorro, and Arija (2016) state in monogastric nutrition (e.g., swine, rats, humans) that grape by-products cause antioxidant and antimicrobial effects when fed to animals; oxidative stability enhanced meat quality and antimicrobial activity reduces pathogenic bacteria while benefiting specific bacterial strains in the gut intestinal tract. The statement is more evident when a study demonstrated weaned pigs displayed diarrhea relief with a heightened immune system (Hao et al., 2015). For ruminants, sheep have been the primary test subject with grape pomace and although they have different digestive systems, observing microbiome changes may foreshadow results in equines. Initially, wine by-products provided valuable sources of energy and protein in ruminants, yet studies involving sheep fed grape pomace showed minimal results, as they receive low energy value from fresh grape pomace; results are variable depending on seed and pulp ratio of pomace (Baumgärtel, et al., 2007; Molina-Alcaide, et al., 2008). Aside from energy value, other observations have depicted loss of volatile fatty acid and linoleic acid which correlates with microbial fermentation loss (Correddu et al., 2015; Guerra-Rivas et al., 2017). Although past studies with sheep indicate adverse results for horses, fermentation location is a keynote. Since equines have a combination of monogastric stomach with hindgut fermentation, interpreting prior studies involving animals like rats, swine, and sheep are consequential to understand effects involving certain areas of the equine gastrointestinal tract.

Grape Residue as Feed for Horses and Hindgut Fermenters

As a wide variety of by-product feeds increase for agriculture animals, there is still a research deficit with in vivo horse nutritional studies. When acknowledging the efficiency of a feed, benefits include reducing feed intake while increasing nutritional digestibility in horses which was tested with dried grape pomace in Slovak warmblood horses (Kolláthová et al., 2020). Twelve adult horses were split into three experimental groups consisting of a control group and two enriched groups. Enriched groups were supplemented with dried grape pomace, primarily comprised of grape skins and seeds with residual grape pulp and fragments of grape stalk. Total tract digestibility of dry matter, organic matter, crude protein, acid detergent fiber, and neutral detergent fiber were measured utilizing total feces collection and lignin as a marker. Overall, there were no health or metabolic problems in horses fed dried grape pomace. Findings from total feces collection demonstrated horses fed 200 grams of pomace showed a positive trend for nutrient digestibility, while horses on the 400-gram pomace diet had negative effects with nutritional digestibility. The authors note flaws in their study and conclude dried grape pomace can be a promising feed component for horses, but further research is required.

Kolláthová et al. produced a study with a better understanding of the nutritional digestibility of dried grape pomace, which is essential if grape pomace is to be considered as a feed additive in the future. An advantage within this study is the amount and uniformity of horses available in the feeding trial. Twelve is a sufficient amount, but when divided into three groups population groups significantly decrease with four horses only receiving a treatment diet. A Latin square design will have greatly expanded these population groups, thus reducing possible outliers, and counterbalancing sequential effects (Bradley, 1958). A notable feature about this study is horses fed grape pomace were all fed a fixed amount (200 or 400 grams), even though base feed (crimped barley and oats, meadow hay, and muesli feed supplement) were all fed accordance to body weight of each individual horse. Depending on weight, smaller horses may have different effects compared to larger horses due to their feed requirements for maintenance, but horses were weight prior to study and maximum standard deviation was 50 kilograms for horses in the control group. Horses in grape pomace groups (200 and 400 grams) had a weight standard deviation of 20 and 30 kilograms in their respective groups, and perhaps weight differences were low enough to not observe significant differences; dried grape pomace enrichment was mixed with their concentrates and was never noted if horses consumed all of their feed, although it was stated that there were no problems with feed intake. Flaws noted by the authors were unreliable coefficients by the lignin markers in the control group and horses fed 200 grams of dried grape pomace; believed to be caused by low amounts fed in smaller feed rations. Overall, dried grape pomace has full potential as a feed additive for horses in certain amounts.

A preliminary research to view the relationship between grape residue and horse performance was conducted in Australia (Davies et al., 2009). A rare study as a feeding trial was administered, utilizing grape seed extract to observe general health, feed intake, and digestion of exercised horses. With only four horses available, a Latin square experimental design was employed with four diets: control, 50, 100, and 150 milligrams of grape seed extract per kilogram of horse's body weight (mg GSE/kg BW) with the treatment groups fed the control diet supplemented with the dosages of grape seed extract. Feeding trial consisted of four 21-day treatment periods alongside 14-day adaptation and 7-day sampling period. Although adaptation and acclimation periods prolong the study they are absolutely required as prior research suggests multiple diet changes within a short amount of time causes digestive issues, with colic being the primary diagnosis (Cohen et al., 1999). Data collected for this study had a wide variety as body weight, temperature, heart rate, respiration rate, blood glucose, fecal pH, and mineral concentration were all analyzed.

A vast array of parameters collected make this study valuable for an introductory project, but the assortment of the small population with uneven ages (ages 5-16 years) of horses do place some constraints; fortunately, the Latin square design expands data potential and uniform breed (Thoroughbreds) reduces a few cofactors. Still, results from this study show valuable precursors towards future research involving equine digestion and nutrition. Significant changes to acknowledge are the fecal pH of treatment groups increased as larger grape seed extract concentrations were supplemented. Control diet, which acted as the base diet for all four treatment groups, consisted of grain-based muesli and was addressed by Davies et al. as a reason for the acidic feces. The control group had the most acidic feces with a pH of 6.6 and gradually rose to 7.0 from the treatment group of 150 mg GSE/kg BW. Change in fecal pH indicates some relative changes in the gut of the horse, specifically the gut microbiome.

Rhinoceros as Hindgut Fermenter

Rhinoceros are hindgut fermenters and have had extended research comparing them to horses. Grape seed extract has been a research concern for captive rhinos as it is believed to be a reliable resource to combat iron deficiency in zoos. Tannins and other polyphenols found in grape residue are capable of increasing iron-binding capacity in rhinos, thus retaining essential iron to maintain healthy physiological functions(Johnson, 2014). To proceed with further research, in vitro studies analyzed microbial changes in captive rhinos and horses fed grape seed extract. The purpose to conduct these studies were to view the effects of the grape seed extract on microbial fermentation of hindgut fermenters and compare digestive patterns in both species, establishing horses as a possible digestive model for further rhinoceros's nutrition research (Huntley, Naumann, Kenny, & Kerley, 2017). Horses and rhinos were compared after incorporation of grape seed extract in four different treatment groups: 0.0, 1.3, 2.7 and 4.0% grape pomace of dry matter (GP DM). Both species' fermentation characteristics demonstrated similar reactions of decreasing characteristics, but to the extent of ammonia, pH, and volatile fatty acid content differed significantly. This study gives insight on possible outcomes when a grape residue interacts with the cecal microbiome of a horse.

In a parallel study with captive rhinoceros, horses inhibited a decline in some fermentation characteristics, most notably ammonia concentration. The inclusion of grape seed extract had a linear decrement of ammonia concentration as more extract was added. In the control diet (0.0 GP DM), initial average ammonia concentration for horses was 8.13 micromoles (mM) and reduced to 4.11 mM with a low grape seed extract treatment (1.3% GP DM). Decline of ammonia concentration continued, eventually reaching .99 mM for horses that 4.0% GP DM incorporated into their diet. Rhinos' ammonia concentration declined as well with the inclusion of grape seed extract, but horses had at least 2.3 times greater concentration than rhinos in each treatment group. Although both species are hindgut fermenters, Johnson elaborates that species differ in their variation of diets; horses are grazers and rhinoceros are browsers, feeding on any forage they can find. Horses initially producing more ammonia have an intricate microbiome compared to rhinos that perhaps have a greater variety of microbe populations, specifically less that produce ammonia. Overall, seeing a decrease in ammonia concentration signals fermentation transformations and with polyphenols having antimicrobial properties, microbial populations decrease resulting in diminution of gas production and volatile fatty acid production (Yu & Ahmedna, 2013).

Ruminant Research

Production animals like cattle, sheep, and goats are vital to the meat and dairy industry, hence they are popular research subjects to maximize yield production while maintaining quality of products. Comprehensive research of ruminal digestion is critical as it is the primary source of nutrients for animals like cattle, sheep, and goats and are believed to be the most successfully evolved herbivores (Clauss, Hume, & Hummel, 2010). Research with grape residue and ruminants are more prominent compared to in vivo studies with horses, which are sparse. Ruminants and horses have microbial digestion (fermentation) incorporated into their gastrointestinal tract, relying on volatile fatty acids excreted from microbes as a nutrient source essential to maintenance and growth. Differences occur in the location of fermentation; ruminants have a compartmentalized stomach (e.g., rumen) where digesta will first enter and ferment, contrast to equids where digesta will travel the through a single chambered stomach, small intestine, and the large intestine. The large intestine contains a chamber called the cecum and this is the fermentation site for equids, hence they are termed hind-gut fermenters to depict their digestive unit. Although different digestive units may not make ruminant species the perfect model for horses, species like cattle and sheep are still heavily relied on to examine microflora changes and hypothesize changes in horse gut microflora.

Sheep

Large sheep populations and compact size make them a valuable resource for animal nutritional research, and although they have different gastrointestinal tracts, their fermentation capability allows them to be a model for equines regarding gut flora patterns. Viticulture by-products has previously been explored as feed for sheep and the consensus is wine by-products are a valuable energy and protein source (Molina-Alcaide et al., 2008). Grape pomace from red wine was evaluated *in vitro* as a feed for sheep in Spain and authors, Guerra-Rivas et al. (2017), discussed pomace's pulp and seed ratio determines the nutritional quality of the feed. Grape pomace with greater pulp ratio contains lower lignified fiber, higher digestibility and higher polyphenol content resulting in greater volatile fatty acid production. Baumgärtel et al. (2007) analyzed a variety of grape pomace and concluded wine processing procedures and grape variety impacts digestibility and energy value of grape pomace for sheep.

Analyzing microflora changes in sheep, whether it be *in vitro*, using fistulated animals or fecal matter, all gather information required to understand changes in the equine microflora. A study conducted in Iran noted sheep organic matter digestibility, dietary fiber digestibility, and rumen protozoa and bacteria all declined when sheep were fed a diet constituting primarily grape pomace (Abarghuei, Rouzbehan, & Alipour, 2010). The large amounts of tannins found in grape pomace were to blame for such a negative a decline, thus Abarghuei et al. had another treatment group where sheep were fed grape pomace with 90 grams of polyethylene glycol to neutralize the high polyphenol concentration. Instead of using grape pomace as a main feed source, administering it as a supplement can improve gut health in lambs (Kafantaris et al., 2017). As previously noted, polyphenols are toxic if not fed responsibly and are recommended as a supplement. The study conducted by Kafantaris et al. found lambs fed grape pomace had a decreased number of *E. coli* in their fecal microbiota alongside pathogenic bacteria. Some forms of E. coli can cause diarrhea and colonic problems in ruminants, and eventually contaminate meats used for human consumption. The study also found beneficial bacteria increased, such as *Bifidobacterium*, that support microbial digestion in fermenters (e.g. cattle, horses). Sheep have demonstrated an overview of how grape pomace is utilized in their fermentation site and can lead to inferences regarding horse microbial digestion of grape pomace, although factors like small intestine digestion and retention time need to be addressed.

Cattle

Cattle are exemplary models for horses due to their sheer size and relation to feed consumption, compared to sheep. Differences to note between horses and cattle are the fermentation capacity in each animal; horses can hold up to 30 liters of feed and fluid while cattle have a capacity of 184 liters, varying on breed (Moore et al., 2001). Both species have different gastrointestinal tracts, but like sheep, obtaining valuable microflora changes in cattle can foreshadow possible changes in the horse's microflora. Environmental changes can alter the microbial ecosystem found in cattle and horses housed in the same property, and introducing a feed substance like grape pomace can produce similar results (Park & Kim, 2019).

Similar to sheep, cattle fed grape pomace had progressive physiological responses. Dairy cows supplemented with an average of 4.5 grams of grape pomace per cow per day in a high protein diet resulted in a limited increase in milk yield and no significant change to protein yield (Nielsen & Hansen, 2004). The study had a hypothesis of improved protein utilization with the supplementation of grape pomace, but was rejected and it is believed to be caused by protein degradation implemented by tannins found in. Another study utilizing sheep and dairy cows found the high fiber, lignin, and tannin concentration found in grape pomace lowered digestibility in fattening lambs. Dairy cows alternatively, had increased healthy fatty acids in their milk compared to standard fodder (Eleonora, Dobrei, Alina, Bampidis, & Valeria, 2014). The micro ecosystem located in the rumen of cattle supplies 60-80% of the energy needed for cattle maintenance and the energy source comes primarily in the form of volatile fatty acids (Hall, 2001). The dietary fiber content in grape pomace promotes healthy microbial digestion and fatty acid production, as evident by the increase of fatty acids in milk. Direct observations of rumen microbiota were altered when grape pomace was supplemented in dairy calves' diet (Biscarini et al., 2018). Common rumen bacteria phyla that prevailed in supplemented calves were Firmicutes and Bacteroidetes, which are common dominant phyla in the horse's cecum as well (Zhao et al., 2015). Diversity grew in the rumen microbiome of the calves, but richness did not significantly change. Emphasizing that equines and ruminants do not have the same gastrointestinal tract, they still provide valuable knowledge between the correlation of grape pomace and gut microbial changes.

Studies involving grape residue and horses have been rather sporadic. For all the fears horse owners have of feeding a fruit-based by-product to their assets due to its potential of high carbohydrate, research has been able to debunk some of these concerns. Research with grape residue and horse nutrition has primarily been split into feeding trials and in vitro studies. Grape seed extract has been the main grape residue utilized for research between grape by-products and horse nutrition. Parameters for previous studies with equids and other species included but not limited to vital signs (e.g., body weight, pulse rate, respiration rate, etc.), feed intake, growth performance, digestibility, gut and fecal microbiota. Overall, patterns indicate high polyphenol count in grape pomace are detrimental to the digestive gut microbiome if given in excessive portions and is evident in not only horses, but animals with fermentation capabilities including ruminants.

Analyzing various research studies with similar goals will model the experimental design for this study consisting of a feeding trial and fecal collections.

CHAPTER 3: IMMEDIATE EFFECTS OF GRAPE POMACE ON HORSE (*EQUUS CABALLUS*) FECAL MICROBIOTA AND PH

3.1 ABSTRACT

Grape pomace is a leading by-product in the wine industry causing many environmental concerns but using it as feed supplement can reduce some of this waste. Grape pomace has many metabolic functions including antimicrobial activity which can be beneficial in equine nutrition. This study studied the effects of grape pomace on the equine fecal microbiota and pH. Seven Arabian horses were used in a cross-over randomized controlled trial with two feeding groups (control and treatment). Treatment diet consisted of a control diet supplemented with dried grape pomace (i.e., stem, skin, and seeds) at .01% of body weight in a 21-day feeding trial. Fecal pH, total bacteria, *Lactobacillus* spp., and methanogens were examined with microbe populations evaluated through fold change analysis. Statistical significance was determined through repeated measures of analysis of variance (ANOVA). Fecal pH had a range of 7.39 – 7.77 with no significance difference between control and treatment group ($p = 0.75$). Logarithmic total bacteria also had no significant difference with fold change ranging within 3.557 to 4.326 ($p = .95$). Fecal *Lactobacillus* spp. and methanogenic microbes also had no significant differences between control and treatment groups. Overall, horses supplemented with dried grape pomace at .01% body weight showed no difference in fecal pH and microbes observed from the control group. Results provide additional information needed to contribute to the limited research of dried grape pomace as a suitable feedstuff for equids.

Keywords: grape pomace; equids; feces; pH; microbiota.

3.2 INTRODUCTION

Global wine consumption remains at a high rate of at least 22 billion liters consumed per year leading to a large worldwide yield of organic waste (Organization of Vine and Wine, 2020). During wine production, roughly 20-30% of the grape is left behind in the form of seeds, skin, and pulp, which compiles to approximately 8.2 million tons of by-product waste worldwide in a single year (Alston et al., 2018; Beres et al., 2017). As organic matter, grape pomace has the versatility to be used in various forms, including as a viable feed source for livestock. Experiments with ruminants, such as cattle and sheep, have shown promise in introducing grape pomace as feed (Guerra-Rivas et al., 2017; Molina-Alcaide et al., 2008). Rich in polyphenols, grape pomace contains antioxidant and antimicrobial properties that can promote healthy physiological function (Chedea et al., 2016). Expanding grape pomace as feed to horses increases the chances of reducing wine industry waste as well as introducing a new feed into the equine industry with possible nutritive benefits.

Horses are hindgut fermenters meaning they have a chamber where large capacity microbial digestion transpires. Before the digesta reaches to the hindgut, feed passes through the foregut which is considered the simple stomach and small intestine, similar to that of a human or pig. In the stomach, feed is mechanically and chemically disintegrated where it will later be absorbed in the small intestine. Feed that cannot be digested in the foregut are a variety of forages and fibrous feeds where the lignin and other cellulose compounds remain unscathed and undigested. Instead, those dietary carbohydrates are fed to a population of microbes in the cecum, located in the colon of the horse. The cecum is the site of bacterial fermentation in the horse where microbial digestion results in the production of volatile fatty acids. These fatty acids are essential to horse nutrition as they comprise 50-70% of overall digestible energy (Glinsky et al., 1976; Vermorel et al., 1997). When introducing a new feed to horses, great consideration is required as disrupting the cecal microbiota can result in dire consequences.

Research with horses and grape residue are limited and are either feed studies viewing specific parameters or *in vitro* fermenters simulating cecal activity. Fecal matter is a suitable observation for assessing the status of the cecum. Feces has its own microbiota that reflects the microbial community located in the cecum (Ericsson et al., 2016). Fecal pH is also an indicator of cecum status as out of range conditions are symptoms of acidosis, a condition where the cecum is too acidic, and the microbial community gets disrupted. This study used fecal pH and certain fecal microbial populations as parameters to study changes in fecal microbiota in horses supplemented with grape pomace. Results will contribute to understanding between grape pomace and horse nutrition, and hypothesis for this study includes grape pomace supplementation affecting fecal parameters measured.

3.3 MATERIAL AND METHODS

All ethical considerations have been approved by the university’s Institutional Animal Care and Use Committee (IACUC).

ANIMALS AND HOUSING

Seven Arabian horses were provided by the W.K. Kellogg Arabian Horse Center from California State Polytechnic University, Pomona. Four geldings and three mares, four years of age, were housed in individual 3.66 x 3.66-meter (13.4 m^2) stalls bedded with fine wood shavings and connected to dirt runways; stalls are adjacent to one another to support natural social behavior. Feeding was conducted thrice a day with alfalfa fed in the mornings (0600 hour) supplemented with *Purina® Strategy Professional Formula GX Horse Feed*, Bermuda hay fed in the afternoons (1100 hour), and evening (1600 hour) feeding replicating morning feeding; forage was accordance to body weight requirement and water and salt block will be provided ad libitum. Horses were exercised approximately 30 minutes a day and maintenance care (e.g., hoof trimming and grooming) managed by the institution's personnel. Feeding guidelines were equated to the Committee on Nutrient Requirements of Horses (National Research Council Board on Agriculture and Natural Resources, National Academy of Sciences).

EXPERIMENTAL DESIGN AND DIET

A cross-over randomized control trial was utilized, constituting a 14-day acclimation period, 21-day control diet, 21-day washout period and 21-day treatment diet. Horses were randomly allocated to either a control or treatment group. For the acclimation period, all horses were housed in their stall and fed the control diet, to get comfortable and accustomed to their environment. Once the acclimation period was completed, four horses remained on the control diet and four horses were fed the control diet supplemented with dried refined grape pomace mixed with applesauce. Applesauce was supplemented to both experimental groups to increase palatability and delivery of grape pomace. After the 21-days, all horses underwent the washout period, where animals revert to the control diet to excrete the grape pomace from their system (relevant to those who were fed grape pomace). Once washout period was completed, a crossover design was implemented with the three horses that were supplemented with grape pomace stayed with the control diet after the and the four remaining horses were supplemented with dried refined grape pomace; all seven animals participated in both diet groups by the end of feeding trial.

Control diet consisted of forage, concentrates, and approximately 113 grams of applesauce adhering to maintenance requirements (minus the applesauce) needed for 4-year-old horses (Table 1). Treatment diet consisted of the control diet with addition of a mixture of approximately 113 grams of apple sauce and 100 milligrams of grape pomace per kilogram of individual horse's bodyweight (mg GP/kg BW). Daily forage and concentrates were fed by horse center's staff and supplementation of applesauce (control) and grape pomace mixture (grape pomace plus applesauce) (treatment) was facilitated by trained research personnel. Uneaten pomace mixture at the time of feeding was mixed in with their concentrate. To calculate dosage, all horses were weighed via a digital livestock scale the day prior to each feeding phase (total of two weigh-ins). Each horse had a specific dosage of grape pomace adjusted to the 100 mg GP/kg BW scale. Supplementation occurred once a day (0700-hour feeding) and each horse had their own individual feeding dish where research personnel fed applesauce (control) and compiled the grape pomace mixture (treatment) to also be fed.

Fecal samples for pH measurements and microbial research were collected weekly within the designated feeding periods (21-day control/treatment diet) omitting any acclimation and washout periods. Fecal collection occurred after supplementation and included research member collecting fecal matter and storing it in a sterile conical centrifuge tube and placed in an industrial freezer (-40°C) until further use; fecal pH was recorded at time of collection utilizing a Oakton® Waterproof 450 pH meter (Oakton

Instruments, Vernon Hills, Il). Horse stables were cleaned of prior excrements before collections, thus giving plenty observation opportunities of multiple stables concurrently. All fecal collections occurred within minutes when horses defecated. Fecal samples were weighed into 12-gram portions and then mixed with 12 milliliters of distilled water, as adhered to recommended standard horse fecal pH measurements (Hydock et al., 2014). pH values were recorded twice and averaged. If pH had a difference greater than 10%, then the sample was measured a third time and averaged with other two measurements.

GRAPE POMACE PREPARATION

Grape residue from a local winery in Riverside County was donated for this study and derived from Cabernet Sauvignon grapes, one of the world's leading grapevines for wine production (This, Lacombe, & Thomas, 2006). Residue was left to sun dry for two weeks, refined, and then grounded up. Nutrient analysis was conducted before study (Table 2) and no mycotoxins were detected (≤ 2 parts per billion) in toxin screening (Cumberland Valley Analytical Services, Waynesboro, PA).

LAB PREPARATION

Deoxyribonucleic acid (DNA) from feces was extracted through the FastDNA™ SPIN Kit (MP Biomedical, Irvine, CA). Deoxyribonucleic acid concentration was determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and then each sample was diluted appropriately to have an equivalent concentration of 50 nanograms per microliter. Three different colonies were observed in the form of the total bacteria in each sample, methanogenic archaea (methanogens), and *Lactobacillus* spp. Deoxyribonucleic acid from the target colonies were amplified via real-time polymerase chain reaction (qPCR) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Temperature and dilution gradient testing were first initiated to find optimal qPCR protocol. Polymerase chain reaction mixtures consisted of 2 microliters (µl) of diluted PCR-ready genomic fecal DNA, 4 µl forward primer, 4 µl reverse primer, and 10 µl of SYBR® Green master mix (Bio-Rad, Hercules, CA). Primers used are available in Table 3. Real-time polymerase chain reaction parameters for testing total bacteria and methanogens are as followed: initial warm up of 5 minutes (min) at 95 °C, followed by 40 cycles of 1 min at 95 °C, 30 seconds (sec) at 60.3 °C, and ending on a melt curve reading from 65℃ to 95℃. The qPCR parameters for Lactobacillus were an initial warm up of 5 min at 95 °C, followed by 40 cycles of 1 min at 95 °C, 30 sec at 55.9 °C, and ending on a melt curve reading from 65 ℃ to 95 ℃.

CALCULATIONS AND STATISTICAL ANALYSIS

Quantitation cycles (Cq) from qPCR were formulated to analyze fold change within the total bacteria colony. Cq difference was first found by subtracting largest total bacteria Cq value to each individual Cq value and the difference was raised to a base of two ($2^{\Delta Cq}$) to acquire total bacteria fold change. The largest total bacteria Cq value used previously was also used to find Cq difference in *Lactobacillus* spp. Cq and generic methanogen Cq, respectively. The largest Cq difference for each respective colony was then used to find fold differences. Fold difference was then raised to a power of two. Statistical significance was determined through repeated measures of analysis of variance (ANOVA) and paired T-test with the Statistical Analysis System (SAS) analytics solutions® (SAS, Cary, NC) under recommendations of (Goni et al., 2009). *P* values less than 0.05 were considered significant.

3.4 RESULTS

Most horses consumed grape pomace mixture instantly when offered to them. Two horses were not interested in the mixture when offered directly but were later consumed when mixed in their concentrate feed and isolated. Overall, all animals took well to the grape pomace mixture.

FECAL pH

Fecal pH was collected on eight separate occasions and results were sorted to their proper week (Week 0, Week 1, Week 2, and Week 3). Initial fecal pH observations (Week 0) were in proximity with the control group starting at 7.47 ± 0.16 and treatment group at 7.56 ± 0.15. During the three-week trial, demonstrated in Figure 1**,** fecal pH ranged from 7.39 ± 0.17 to 7.77 ± 0.16. Control group had a small spike in fecal pH after the first week and decreased back down near the initial week. Fecal pH from the treatment group slowly declined for the first two weeks and increased towards the last week almost to the initial fecal pH. Statistical analysis demonstrated that both control and treatment group had no significant change in fecal pH ($P = 0.75$).

MICROBIAL POPULATION

After eight collection days, 56 fecal samples were collected but due to limited resources, only 42 fecal samples were processed in the laboratory. These samples formulated analysis comparing the initial day with the first- and second-week collections (omitting the third week).

Fecal DNA concentration was in the range between 1.136×10^{-5} micrograms of DNA per gram of fecal sample (µg DNA/g FS) and 1.665×10^{-5} µg DNA/g FS. Control group remained steady in the initial and first week with a fecal DNA concentration of 1.327×10^{-5} µg DNA/g FS ± 1.987×10^{-6} µg DNA/g FS and 1.333×10^{-5} µg DNA/g FS ± 1.736×10^{-6} µg DNA/g FS, respectively. Week 2 concentration was higher resulting in 1.665×10^{-5} µg DNA/g FS ± 1.220×10^{-6} µg DNA/g FS, which is about a 25% increase. The treatment group had a lower week 0 fecal DNA concentration than the control group with the initial concentration starting at 1.136×10^{-5} µg DNA/g FS ± 2.316×10^{-6} µg DNA/g FS. Treatment group differs from the control group in that the treatment group had a spike after the first week and decreased towards the last week as shown in Figure 2. The treatment group's fecal DNA concentration for week 1 was 1.556×10^{-5} µg DNA/g FS ± 2.210×10^{-6} µg DNA/g FS and decreased to 1.336×10^{-5} µg DNA/g FS ± 9.827×10^{-7} µg DNA/g FS by week 2. Statistical analysis showed no significant difference between fecal DNA concentration of control and treatment group ($P = 0.46$).

Fecal total bacteria fold changes within this study ranged from 3.557 to 4.326. Figure 3 demonstrates both control and treatment groups had similar initial fold changes with both starting at 4.258 ± 0.174 and 4.074 ± 0.429, respectively. After one week, the control group stayed near the initial value with a slight increase while the treatment group declined to 3.660 ± 0.391. Two weeks after the initial fold changes, both groups decreased with the control group falling below the initial value towards 4.081 ± 0.192 and the treatment group decreasing to 3.557 ± 0.332. Overall, there is no statistical difference for total bacteria fold changes between control and treatment group ($P = 0.95$).

Fecal *Lactobacillus* spp. fold change ranged between 0.254 and 0.534, with the control group week 0 being the smallest value and all other samples being greater than .400 fold changes. As stated earlier, the control group possess the smallest value at week

0 with 0.254 ± .055 fold changes. Treatment group had initial fold changes of 0.439 ± .020 and stayed nearly the same for week 1 until increasing to 0.534 ± .050 fold changes for week 2 as depicted in Figure 4. Control group's fold changes would increase nearly to the same value as the treatment group after week 0; *Lactobacillus* spp. fold changes for week 1 and week 2 in the control group were 0.450 ± .054 and 0.443 ± .073, respectively. Statistical analysis depicts that there was no significant change in fecal *Lactobacillus* fold change between the control and treatment group (*P* = .33).

Fecal Methanogen fold change within this study had a range from 0.591 and 1.278. Control group started with an initial 1.278 ± 0.274 fold changes and decreased to 0.701 ± 0.207 only for it to go back up to 1.115 ± 0.180 fold changes. Figure 5 shows the treatment group had an opposite pattern where it started low, 0.767 ± 0.154 (similar to control group's week 1 fold changes), spiked to 0.767 ± 0.154 and decreased back to a lower value, 0.591 ± 0.119 fold changes. With all these increases and decreases they were still within a certain range and statistical analysis demonstrates there was no significant change in fecal methanogen fold change (*P* = .61).

3.5 DISCUSSION

This study attempted to evaluate immediate changes in horse fecal microbiota by observing fecal pH, DNA concentration in fecal sapmles, and fold changes from PCR amplification derived from three different microbe primers of two different experimental groups. Fecal DNA concentration was not affected by the supplementation of dried grape pomace, as well as fecal total bacteria count. Microbe diversity within a horse's cecum vastly changes with diet and environment, thus replicating approximate data from previous studies or utilizing animals from different facilities is unfeasible (De La Torre et al., 2019; Julliand & Grimm, 2017). Within the confines of this study, the general pattern that the control and treatment group having similar fecal microbiota on the accountability of species richness and diversity is expected. Bacterium such as *Firmicutes* and *Bacteroidetes* are expected to dominate the microbe population followed by protozoa and archaea, so expanding the analysis of other microbe colonies will have justify the greater effect of dried grape pomace (Julliand & Grimm, 2016; E. Venable et al., 2016). Results demonstrated supplementation of dried grape pomace at .01 percent body weight had no immediate effects on fecal pH, DNA concentration, and total bacteria, *Lactobacillus* spp., and methanogen populations.

Fecal pH is an adequate indicator of internal gut health, and by observing fecal pH in this study, dried grape pomace can be evaluated for immediate effects on the cecum (hindgut) of the horse. Horses in the control group averaged a fecal pH of 7.58 and this can be acquainted to the breed (Arabian), age (four years), and high forage/fiber to concentrate ratio diet that is fed on a daily basis (Kauter et al., 2019). Horse fecal pH collection protocol was followed under suggested guidelines by Hydock, Nissley, and Staniar (2014) which includes the addition of an equal proportion of distilled water. Water was added to insure sufficient fecal liquid was able to submerge the pH meter electrode, but unfortunately Hydock et al. state this action causes greater variation in fecal pH detection. Other factors influencing fecal pH are external/environmental temperatures and data was obtained in a barn in the mornings of the fall season of southern California; optimum temperature for fecal pH collection should be in 20 to 23°C (room temperature). The time from defecation and pH measurement varied between each sample, but the range was between 5 – 30 minutes and measuring within this range was an advantage as there is greater variation in fecal pH within the first three minutes after defecation. Overall, the addition of dried grape pomace to the horse's diet did not change their pH within two weeks as the pomace consist primarily of fibers and polyphenols, compared to grains or other concentrates which can alter the cecal microbiome of horses due to high carbohydrates and reflected in their feces (Fernandes et al., 2014; Glunk et al., 2013).

Fecal pH results do not align with a previous study from Davies et al. (2009), where fecal pH became more alkaline as horses were fed higher doses of grape pomace; 50, 100, and 150 mg GP/kg BW. The horses in that study were training horses and they had more acidic feces (< 7) in their control group and the increase in pH in their treatment groups were .1 for each increment of grape pomace fed. The comparison opens the discussion if grape residue is effective under certain fecal conditions or if grape seed extract has different effects. When evaluating the grape residue supplementation at .01% body weight, the conditions of the control diet had an influence in the overall results. Body weight was used to formulate the supplementation doses, but feed intake was not incorporated for this study. Horses are fed 1.5 – 3.0% of their body weight with nutrient requirements dispersed within that feed ration (Duberstein and Edwards, 2009). Proportioning the grape pomace with the other feeds included in the control diet (Table 1) can further elucidate the relationship between grape pomace and horse nutrition.

Lactobacillus spp. are common microbes found in the hindgut of horses which is responsible for producing lactic acid 90% of the time (Feiner, 2006). Lactic acid (along with the volatile fatty acids) is what establishes the acidic environment of the cecum, then reflected in fecal matter. Adjusting a horse's diet greatly influences how the microbial population will change with previous studies inferring greater concentration ratio results in *Lactobacillus* spp. population growth within a week (Julliand & Grimm, 2017; van den Berg et al., 2013). Fecal *Lactobacillus* spp. and pH are often correlated, thus when fecal pH remained indifferent from both treatment groups, fecal *Lactobacillus* spp. also remaining unchanged is not surprising. There are occasions where fecal microbial populations change but fecal pH shows no significant change such as the study conducted by van den Berg et al. Both this current study and van den Berg et al. were observing immediate changes in fecal pH within a week or two, so it can be possible that changes in fecal pH can be noticeable in longer time.

Dried grape pomace used for this study consisted primarily of fibrous compounds making it not much different than the hay and forage the horses already eat, but the high polyphenol content could have potentially changed fecal parameters of horses in this study. Previous *in vitro* fermentation studies suggest that about 20% of dry matter from grape and other fruit pomaces are digested by common horse fecal microorganisms (Hwang et al., 2017). Even with supplementation of .01 percent body weight of dried grape pomace, an estimated 20% of that matter was digested by the animals, making the amount very minimal and the reason why there was no change in fecal parameters measured in this study. Yet, a previous study supplementing dried grape seed extract at 50, 100, and 150 milligrams of grape seed extract per kilogram of horse body weight did observe increase in fecal pH at 100 milligrams per kilogram of body weight (.01%), but those animal were fed a high concentrate feed of muesli (Davies et al., 2009).

Many research projects tend to focus on training horses that have a large concentrate ratio in their diet, such as Davies et al., and this makes horses in those facilities to have a more acidic cecum (<7.0) making them more sensitive to microbial changes, especially being near the state of acidosis (<6.0). Horses for this study were reserved for research use and were not competitively trained or heavily worked, thus their diet was composed of high forage with concentrated supplementation of a maintenance mix to support weight gain if needed. A majority of polyphenol intake are metabolized in the large intestine by bacterial enzymes (Brenes et al., 2015; Lipinski et al., 2016). Animals fed a high starch diet possess microbes ready breakdown the structural carbohydrates found in fibrous feeds (e.g., grape pomace) and is correlated with acidic feces (Harlow et al., 2015). Perhaps increasing energy use and intake in horses may trigger different effects to the fecal microbiota (Willing et al., 2009).

Methanogens are a community of Archaea that produce methane as by-products in environments that are limited in oxygen. The biogases released by animals (primarily ruminants) have been a concern for global climate change and controlling the anaerobic chambers within animals may assist in combating climate change. Horses' methane production occurs in the hindgut where diverse methanogenic archaea inhabit the cecum and feed of methanol and simple methylated compounds to produce methane (Murru et al., 2018). This study analyzed all fecal methanogenic genera which is why there probably was a higher population count compared to *Lactobacillus* spp. Observing if dried grape pomace has any effects on the methanogen population can potentially have some ramifications for combating climate change, but longer trials will be needed as horses supplemented with dried grape pomace showed no difference in their fecal methanogen population. Phylogenetic analysis indicates equine fecal matter can portray the overall community present in horse digestive tracts, but not the dynamic of changes across digestive tracts (Misiukiewicz et al., 2021).

Limitations within the study arose when considering the size and behavior of the experimental group (horses). Incipiently, a larger number of horses would be preferred to increase sample size of fecal matter and better affirm a stable microbiome within the confined facility. Albeit not feasible, a lack of uniformity presents itself as a limitation; different horse weights require different amounts of feed and concentrate supplementation resulting in an array of fecal microbiotas that may not correlate with grape pomace supplementation. Having access to feed scales and measure each feed ration for each individual horse and proportion the dried grape pomace according to their nutrient intake would have been ideal. Although, change of fecal microbiota is the dependent variable measured, so similar observable changes will negate previous concern. Furthermore, horses will not be observed continuously, so there is the possibility for horses to consume unintended feed provided from unaware visitors and plants that unexpectedly grew around their paddocks, affecting their fecal microbiota.

Limited resources prevented the microbial analysis of the third week which minorly changed the scope of this study into the immediate effects of dried grape pomace. Gut microbiome changes from subtle feed changes may not be observable until 14-21 days of feeding but as noted by previous studies where diets are abruptly changed (e.g., changing forage to concentrate ratio or converting from pasture grazing to stall feeding), there is prone to be change. Gathering other grape residue feed studies, starting with a low dosage of dried grape pomace is not detrimental to horse’s gut health in the immediate future.

Results from this study exhibited dried grape pomace has no instantaneous effects on fecal parameters measured. Focusing on fecal parameters was an introductory approach to test the vitality of dried grape pomace as a horse feed. Fecal microbiota does not necessarily reflect the hindgut microbiota but noticing severe changes in the feces constitutes some disruption in the gut. To further illustrate the potency of grape pomace, other physiological parameters should be measured in blood, weight, and feed intake.

Table 1. Control diet fed to 4-year-old Arabian horses at Kellogg Arabian Center.

Time	**Morning (7 AM)**	**Noon (12 PM)**	**Evening (4 PM)**
Diet	Alfalfa Hay (~5 – 6 kg) Pelleted Feed (~1 – 3 kg) Applesauce (113 g)	Bermuda Hay (~4 – 5 kg)	Alfalfa Hay (~5 – 6 kg) Pelleted Feed (~1 – 3 kg)

Pelleted feed: Purina® Strategy Professional Formula GX Horse Feed; kg = kilograms; g = grams

Table 2. Dry matter nutrient analysis of dried Cabernet Sauvignon grape pomace conducted by Cumberland Analytical Services**.**

Analysis	**Grape Pomace**	**Crude Protein**	**Adjusted Proteins**	**Acid Detergent Fiber**
% Dry Matter	94.4	13.3	13.3	29.7
Analysis	**Lignin**	**Crude Fat**	**Total Digestible Nutrients**	**Non-Fiber Carbohydrates**
% Dry Matter	24.47	9.49	59.35	33.73

Table 3. Primer pairs used in real-time polymerase chain reaction

Primer	**Sequence (5' - 3')**
Total Bacteria	F - TCCTACGGGAGGCAGCAGT R - GGACTACCAGGGTATCTAATCCTGTT
Lactobacillus spp.	F - GCCCAACTGATATGACGTGC R - GCCCATCCTGGAGTGATAGC
Methanogen	F - TTCGGTGGATCDCARAGRGC R - GBARGTCGWAWCCGTAGAATC

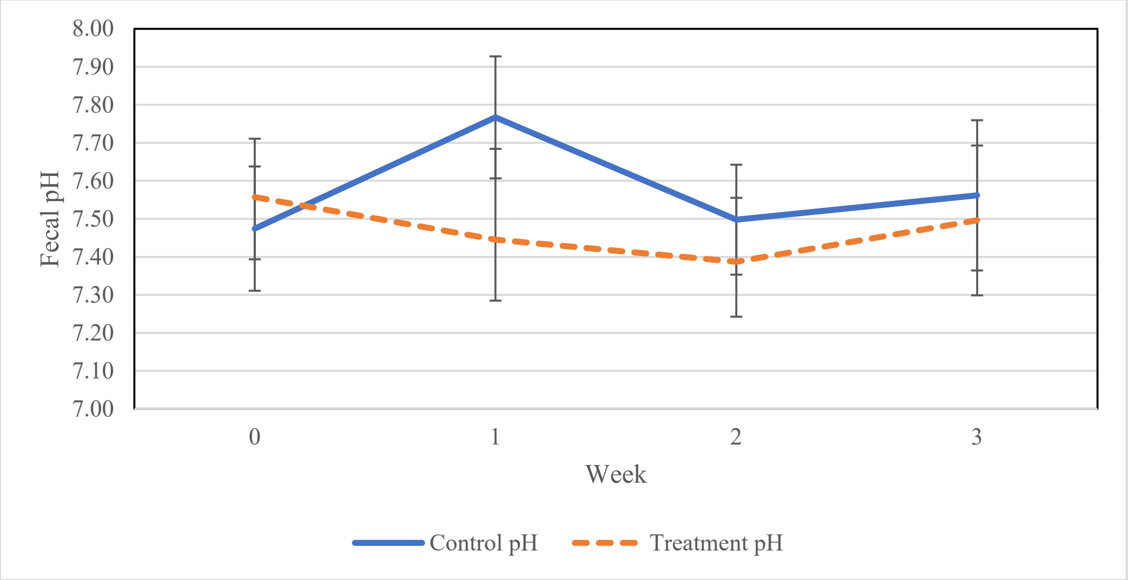

Figure 1. Fecal pH in horses supplemented with dried grape pomace. Treatment group supplemented with .01% body weight of dried grape pomace. P value = .75.

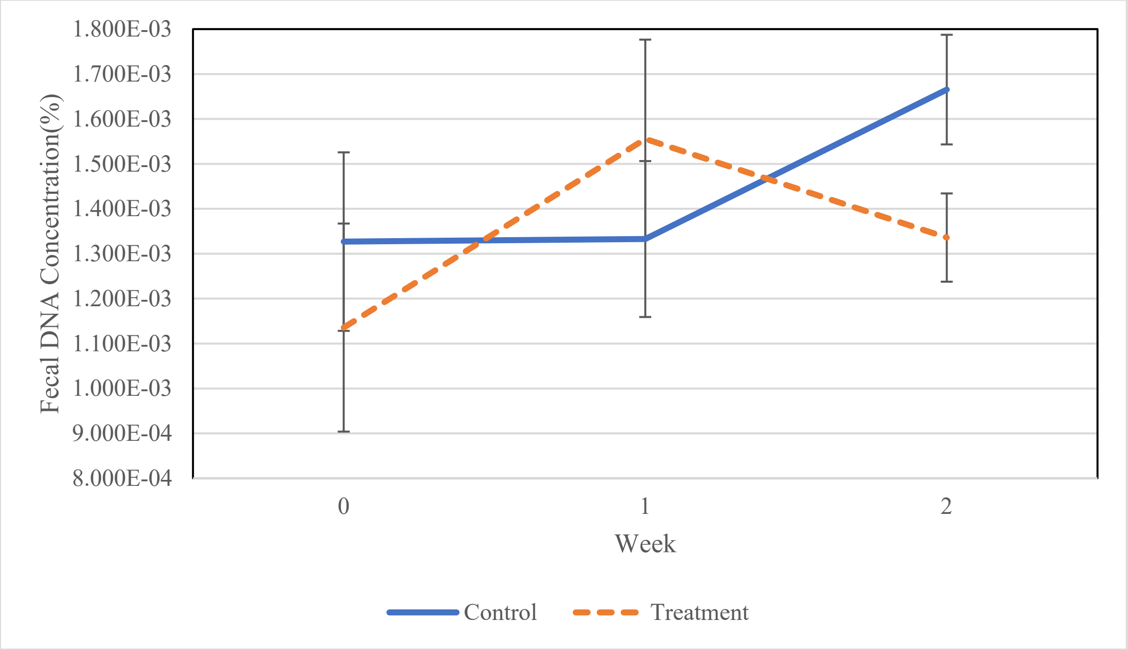

Figure 2. Fecal DNA concentration in horses supplemented with dried grape pomace. DNA, deoxyribonucleic acid. Treatment group supplemented with .01% body weight of dried grape pomace. *P* = 0.4555

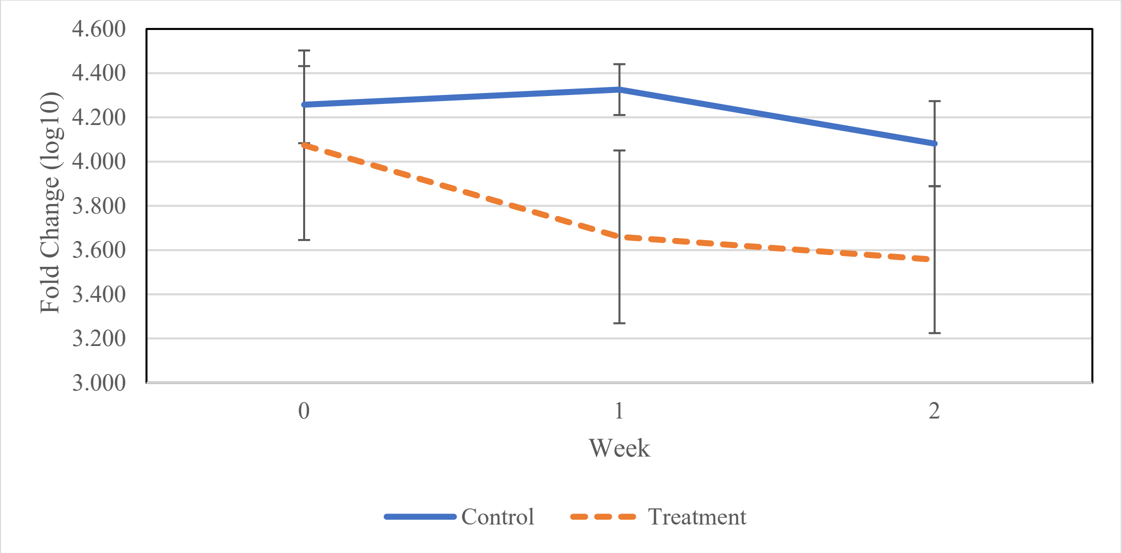

Figure 3. Total bacteria log transformed fold change in horses supplemented with dried grape pomace. Treatment group supplemented with .01% body weight of dried grape pomace. *P* = 0.9475.

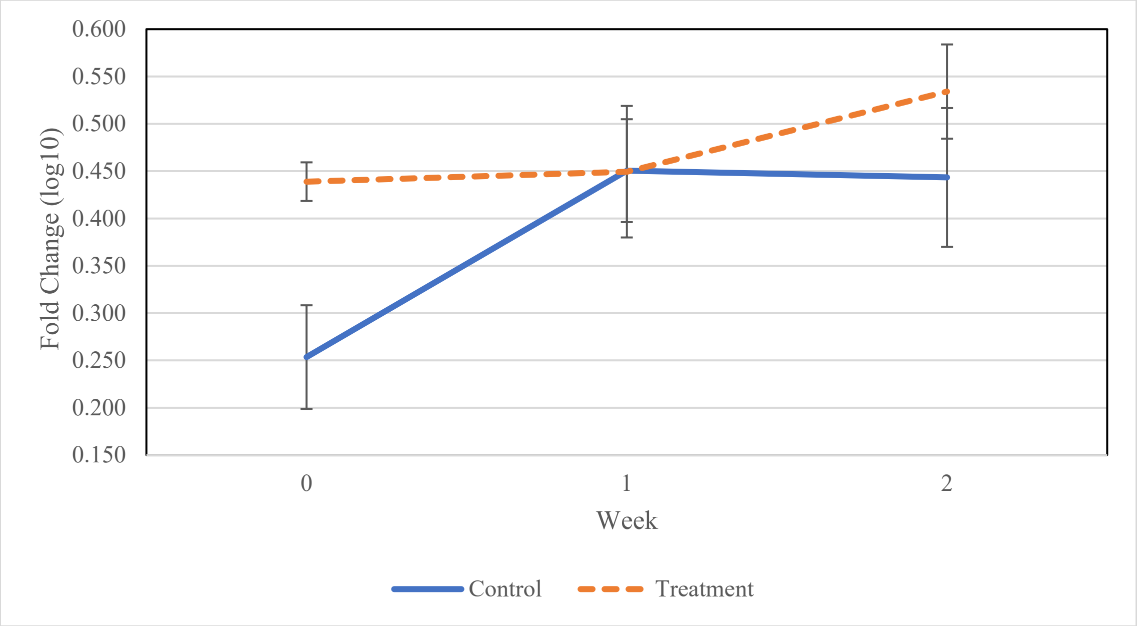

Figure 4. Fecal *Lactobacillus* spp. log transformed fold change in horses supplemented with dried grape pomace. Treatment group supplemented with .01% body weight of dried grape pomace. *P* = 0.3317.

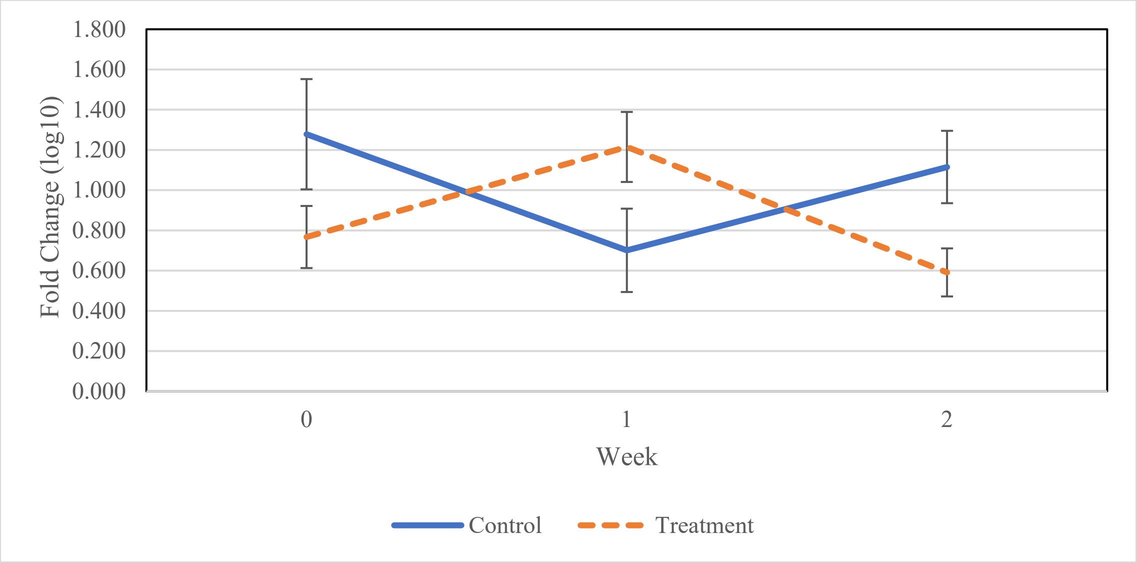

Figure 5. Fecal methanogen log transformed fold change in horses supplemented with dried grape pomace. Treatment group supplemented with .01% body weight of dried grape pomace. *P* = 0.6147.

CHAPTER 4: CONCLUSION

Intentions for the study were to note immediate fecal changes (within three weeks) in horses supplemented with a 100 mg GP/kg BW of grape pomace. By observing fecal pH in three weeks and total bacteria, *Lactobacillus* spp., and methanogen colonies in two weeks, grape pomace had no immediate effects on Arabian horses of four years of age. Fecal pH results did not align with a previous study that saw feces become more alkaline as dried grape seed extract was supplemented, but different initial fecal pH values and supplement raise more potential study opportunities (Davies et al., 2009).

No changes may have been observed in this study, but a greater understanding is formulating about grape pomace as a horse supplement. A prior study observed nutrient digestibility in horses supplemented with 200 and 400 grams of dried grape pomace, where positive trends of nutrient digestibility were recorded until 400 grams of pomace were supplemented. Grape pomace has potential as a beneficial supplement, although the appropriate amount still must be discovered. This study at least finds that feeding 100 mg GP/kg BW is not detrimental to horses in the immediate future after supplementing.

SIGNIFICANCE AND IMPACT

Results from this study will not determine if grape pomace will be the next feed for equines, but rather commend further research to continue the evaluation of grape pomace as an alternative feed or supplement. This study showed no effects between total bacteria, fecal pH, and *Lactobacillus* spp., adding to the limited research of grape pomace is innocuous to horses (with further studies required to support the claim). Findings from the study will act as a safety checkpoint in utilizing grape pomace as feed for horses.

Plenty of previous research replicate pomace digestion in equines by using labs to analyze microbial response in a petri dish or test tube (otherwise known as *in vitro*). Implementing a feeding trial, microbes can behave in their natural environment and the outcome can support or dispute previous findings that used an *in vitro* model.

Furthermore, a goal for this study is to trigger future research relating to pomace and horse nutrition. Anticipated studies will need to track pomace digestion in the fore- and hindgut to understand patterns and mechanisms before feed reaches the cecum. Observing polyphenol physiological reactions from grape pomace will also be a conjugate research topic since grape pomace is rich with such compounds (Yu & Ahmedna, 2013). Eventually, grape pomace can be observed for bioavailability and health benefits in horses, such as reducing inflammation, joint pain and controlling laminitis, due to polyphonic compounds found in the by-product (Zholobenko, Mouithys-Mickalad, Modriansky, Serteyn, & Franck, 2016).

A more subtle impact this research provides is gathering information for endangered animals, such as black rhinoceros (rhinos). Rhinos are hindgut fermenters and have identical gastrointestinal tracts to equines. Using horses as a digestive model can increase feed versatility for species like rhinos and tapirs. Black rhinos in captivity have a common health issue of iron overload disorder, where excessive iron is accumulated in body tissue leading to greater vulnerability to diseases (Johnson, 2014). Polyphenols have the capability to control and prevent this disorder, but as an endangered species, researching has become rather difficult. Fortunately, horses are the ideal model and research has shown black rhinos and horses react the same to limited amounts of grape seed extract (Huntley et al., 2017). Findings from this study promote research for endangered species with hindgut fermentation.

Ultimately, introducing grape pomace into the horse industry will reduce waste produced from wine manufacturing. Millions of metric tons of grapes are harvested annually for goods (e.g. wine, juice, jams) and the leading by-product is grape pomace (Beres et al., 2017). Using pomace as a feed will remove some of this waste from landfills and instead support livestock around the world. Since 2017, 3% percent of grape pomace from wineries is used for animal consumption and research using other species of livestock and poultry have shown benefits from grape pomace supplementation; adding horses to that list can hopefully increase the utilization of grape pomace from wine production (Kalli et al., 2018). With by-products relatively available, grape pomace can also be an affordable product with high nutritional value to many ranchers.

This study demonstrated the viability of specific equine microflora when grape pomace is fed in low dosage, introducing many opportunities for research to utilize grape pomace as a nutritive feed while aiding environmental sustainability by managing waste in the wine industry.

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