Effect of exogenous enzymes on ruminal fermentation, pelletability and palatability of blueberry products in equine feed, and the effects of quantity of protein and starch on equine cecal environment

by

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Abstract

Enhancing ruminal fermentation of feed is an important consideration for producers of beef and dairy cattle. One method for enhancing fermentation of fiber is application of exogenous fibrolytic enzymes (EFE) to feed. To determine whether method used to apply EFE impacts fermentation parameters by bovine ruminal microorganisms, 3 methods of applying Vista Pre-T (AB Vista, Inc., Plantation, FL.) on a total mixed ration (TMR) were evaluated: sprayed liquid enzyme (wet), liquid enzyme combined with molasses (molasses) prior to mixing molasses into TMR, and dried enzyme added to vitamin and mineral premix (dry) prior to mixing vitamin and mineral premix into TMR. Five grams (dry matter; DM) treated TMR, which were calculated to contain 4.82 mg (DM) Vista Pre-T, were placed into fermentation bottles. Additionally, there also were cultures that received 5 g (DM) untreated TMR with 2.41 mg (DM) liquid Vista Pre-T dosed directly (direct-dosed) into fermentation bottles at the time of inoculation. Negative control cultures contained 5 g (DM) untreated TMR and were not exposed to EFE. One hundred twenty-five milliliters McDougall's buffer and 25 mL ruminal fluid, which served as inoculum, were combined with TMR in fermentation bottles. Bottles were sparged with N_2, fitted with gas pressure monitoring modules, and incubated for 48 h at 39°C. Molasses enzyme application reduced maximum rate of gas production and increased time to reach half maximum gas production compared to all other applications ($P \leq 0.05$). Wet application led to greater terminal pH compared to all other treatments ($P \leq 0.05$). Cultures containing molasses treatment had greater ($P \leq 0.02$) terminal pH compared to dry and direct-dosed treatments. Concentrations of total VFA, acetate, propionate, isobutyrate, and isovalerate were not affected ($P \geq 0.12$) by application method. Acetate:propionate ratio was increased when cultures contained molasses treatment compared to direct-dosed and negative control cultures ($P \leq 0.01$).

Butyrate concentrations were greater when cultures contained molasses compared to all treatments ($P \leq 0.009$) except dry ($P = 0.07$). Molasses and dry application methods resulted in greater valerate concentrations ($P \leq 0.03$) compared to cultures containing wet treatment. Dry application led to greater caproate concentrations compared to all treatments ($P \leq 0.02$) except molasses ($P = 0.40$). *In vitro* dry matter disappearance (IVDMD) was greater for cultures containing dry and molasses treatments than all other application methods ($P \leq 0.0009$). Neutral detergent fiber disappearance (NDFD) was greatest for cultures containing dry application ($P < 0.0001$) with wet application method yielding the lowest NDFD ($P < 0.0001$); however, ranges observed for all parameters were small. While molasses treatment had the greatest impact on dry matter disappearance *in vitro*, this effect has not been confirmed *in vivo*.

The objective of this study was to evaluate the impact of liquid blueberry juice (BJ65) or blueberry puree (BP30) used as a binding agent on pellet durability and palatability of a typical equine concentrate when included at 4% of the pellet. Molasses was used as a control. Production data, pellet durability, and moisture content were evaluated in 1 replicate for each treatment. Because moisture content of condition mashes was 17.59% and 18.11% for BJ65 and BP30 treatments, respectively, greater inclusion of blueberry product would likely cause roller slippage and complicate the pelleting process due to increased liquid. Pellet durability met industry standards for all treatments. Pellets were fed in a 3-period crossover study to 9 two-year-old horses to determine the effect of blueberry products on acceptability. All animals were allowed 10 min to consume 1.36 kg at 0700 h and 1700 h each day for 3 d. Consumption time and amount consumed were recorded to calculate intake, intake rate, and intake ratio (IR). No horses consumed all pellets within the allotted time, and thus, treatment differences for intake and intake rate were the same. Period tended to impact intake ($P = 0.0909$), with horses

consuming less during period 1 than period 3 ($P = 0.0317$), but period had no effect ($P = 0.2881$) on IR. Treatment influenced intake ($P < 0.0001$), with decreased intake of BP30 compared to control and BJ65 ($P \leq 0.0001$). Intake ratio was greater ($P = 0.0075$) for BJ65 than BP30 with IR of 0.5069 and 0.4227, respectively. Because IR of 0.50 indicates equal consumption of treatment pellets compared to control, consumption of BJ65 was no different than control. Thus, BJ65 appears to be more acceptable to horses than BP30 when included in dietary pellets at this rate.

Fluctuations in relative abundances of microorganisms in the equine hindgut have been associated with colic, and, while equine diets contain varying ratios of forage:concentrate, little is known regarding effects of increasing dietary starch on the microbiome of the equine hindgut. Thus, an experiment was conducted with six cecally cannulated horses (524 ± 65.5 kg BW) to evaluate effects of increasing dietary starch on equine cecal microbiota. Starch was supplied via pelleted corn and increased by 0.5 g starch·kg BW^{-1}·$meal^{-1}$ every 7 d until horses received 3.5 g starch·kg BW^{-1}·$meal^{-1}$. Smooth bromegrass hay and water were offered *ad libitum*. Meals were fed every 6 h, starting at 0600 h. On d 7 of each period, cecal digesta were collected every 2 h for 12 h, with the h 0 collection occurring prior to the 0600 h feeding. Cecal samples obtained from all time points for a given level of dietary starch within an individual horse were pooled, DNA was extracted for PCR amplification of the 16S rRNA gene (V3 and V4 regions), and sequencing was performed using an Illumina MiSeq. Mothur was utilized for clustering of features and operational taxonomical units (OTUs), and sequences were submitted to SILVA database for identification. Data were analyzed (SAS version 9.4) as a completely randomized design with fixed effect of treatment (g starch·kg BW^{-1}·$meal^{-1}$) and random effect of horse. Across treatments, Firmicutes was the most abundant phylum, followed by Bacteroidota. Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ elicited the greatest changes in microbiota, indicated by decreased ($P \leq$

0.0469) relative abundances (RA) of *Rikenellaceae*, *Prevotellaceae*, *RF16 group*, *Spirochaetaceae*, *Alloprevotella*, *Prevotella UCG-003*, *Prevotella UCG-004*, *RF16 group* genus, and *Treponema* compared to all other treatments. Conversely, feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in increased ($P \leq 0.0045$) RA of *Christensenellaceae* and the *R-7 group* genus compared to all other treatments. If a horse presented with symptoms of colic, it was removed from the experiment. Data obtained when feeding 0.5, 1.0, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ were compared between horses that completed the trial and those removed using a covariate of 0 g starch·kg BW^{-1}·$meal^{-1}$. When consuming 0 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted had greater ($P \leq 0.00454$) RA of *Aeromonadales*, *Succinivibrionaceae*, and *Selenomonadaceae* compared to horses that were removed. When feeding 0.5 g starch·kg BW^{-1}·$meal^{-1}$, no differences in RA of taxa were detected between horses that persisted and horses that would later be removed. Horses that were removed had greater RA of *Colidextribacter* ($P = 0.0057$) compared to horses that persisted when feeding 1.0 g starch·kg BW^{-1}·$meal^{-1}$. When consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted had greater ($P \leq 0.0500$) RA of *Negativicutes*, *Acidaminococcales*, *Acidaminococcaceae*, *Phascolarctobacterium*, and *Ruminococcus* compared to horses that were removed. This is one of the first reports describing effects of gradually increasing dietary starch on equine cecal microbiota *in vivo*. As well, this is the first report to compare cecal microbiota of horses tolerant of increasing dietary starch to those susceptible to colic in response to such dietary challenge.

Dietary protein recommendations for equines are not consistent and may not account for microbial nitrogen requirements in the equine hindgut. To assess the impact of nitrogen on fermentation by equine cecal microorganisms, cecal fluid from 4 cecally cannulated horses was used to inoculate fermentation bottles containing buffer, forage, and supplemental nitrogen. In

experiment 1, sodium caseinate (SC) provided 0, 0.5, 1, 2, or 4% additional CP to bottles containing alfalfa (22.4% CP) or native warm season prairie grass hay (4.8% CP). Bottles were equipped with continuous gas pressure monitors and placed into a shaking incubator for 48 h at 39°C. Cultures with alfalfa had greater ($P < 0.0001$) *in vitro* dry matter disappearance (IVDMD), NDF disappearance (NDFD), ADF disappearance (ADFD), cumulative gas production and total VFA than those with grass hay. All levels of sodium caseinate increased gas production ($P \leq 0.05$) and decreased pH ($P < 0.003$) in cultures with grass hay. Sodium caseinate at 1, 2, or 4% additional CP increased IVDMD, NDFD, and ADFD ($P < 0.01$), while 4% additional CP also increased total VFA ($P < 0.01$) in cultures with grass hay. For experiment 2, SC, fishmeal, soybean meal (SBM), whey, porcine blood plasma, and L-lysine hydrochloride were added to supply 2% additional CP equivalent to cultures with grass hay. All nitrogen sources decreased pH and increased IVDMD, NDFD and ADFD ($P \leq 0.01$), with the largest effects elicited by SC, L-lysine, and whey ($P \leq 0.05$). Total VFA ($P \leq 0.04$) and gas ($P \leq 0.05$) production increased with L-lysine, whey, SC, SBM, and fishmeal. While nitrogen supplementation had minimal effects on cultures containing alfalfa, it altered fermentation and increased digestibility, as measured by IVDMD, NDFD, and ADFD, of grass hay, more notably with more soluble protein sources.

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Dedication

I dedicate this thesis to my mother, Sher Halpin. Thank you for teaching me how to dream big and always encouraging me to follow those dreams. I did it.

Preface

Chapter 7 has been published in the Journal of Equine Veterinary Science (JEVS). In accordance with journal requirements, citations and section headings for this chapter are numbered in order of appearance.

Chapter 1 - Literature review: Ruminal fermentation and exogenous fibrolytic enzymes

Introduction

There is a need to increase efficiency in beef and dairy cattle production to meet the nutritional demands of the growing human population while using fewer resources. With less land available for grazing, improving feed efficiency, or ability of cattle to convert feedstuffs to usable product, is a primary goal of producers (Koch et al., 1963; Blake and Custodio, 1984; Osterburg et al., 2010). Enhanced feed efficiency leads to greater milk production or weight gain, with decreased inputs and/or increased outputs for the producer (Arthur and Herd, 2005).

Ruminal fermentation is an important consideration when feeding cattle, as feed will first be subjected to microbial fermentation and utilization, which alters the availability of nutrients to the animal. Microorganisms convert structural carbohydrates (sCHO), which are not digested by mammalian enzymes, to volatile fatty acids (VFA) in the rumen (Wang et al., 2001; Wang and McAllister, 2002). These VFA can be utilized by the animal as a source of energy (Bergman, 1990). Maximizing fermentation of sCHO while limiting microbial degradation of lipids, protein, and non-structural carbohydrates (nsCHO) is of interest to ruminant nutritionists and producers (Garg, 1998).

Application of exogenous enzymes (EE) to feedstuffs has been used to enhance ruminal fermentation. Exogenous enzymes are derived from ruminal microorganisms and applied to rations prior to feeding (Beauchemin et al., 2003; Adesogan et al., 2007). While proteolytic, saccharolytic, and lipolytic EE are available, exogenous fibrolytic enzymes (EFE) are the most utilized EE as they increase ruminal fermentation of sCHO (Beauchemin et al., 2004a). There are

2 predominant theories regarding mode of action of EFE. The first is that pre-ingestive hydrolysis of fiber occurs (Wang et al., 2001). The second theory is that there is a synergistic relationship between native ruminal microorganisms and EFE (Morgavi et al., 2004). It is likely, however, a combination of both pre-ingestive and ruminal effects result in increased microbial fermentation of fiber and enhanced animal production when EFE are utilized in cattle rations.

Microbial fermentation of nutrients in cattle will be covered in this review, focusing primarily on fermentation of fiber. The effect of common feed components and methods for enhancing microbial fermentation of fiber will be explored with emphasis on EFE. Lastly, the effect of EFE on animal performance, digestibility of nutrients, and variables impacting these effects will be included.

Digestive physiology

Cattle are ruminating herbivores, having a large 4-chambered stomach consisting of the reticulum, rumen, omasum, and abomasum (Membrive, 2016). Both microbial fermentation and mammalian enzymatic digestion occur within the 4-chambered stomach. Microbial fermentation is of particular importance, as cattle do not synthesize the necessary enzymes to degrade fiber. Ruminal microorganisms produce cellulases and xylanases, which break down poor-quality forages and yield VFA, ammonia, and amino acids (AA). These end products provide nutrients for microbial cellular maintenance and proliferation, and energy for the animal (Russel and Wilson, 1996; Wang et al., 2012). Following fermentation, feed is subjected to enzymatic digestion in the abomasum and small intestine. Any remaining undigested or unabsorbed nutrients will flow into the large intestine where they will undergo hindgut fermentation. Hindgut fermentation of carbohydrates (CHO) accounts for 5 to 10% of total tract digestibility of fiber in dairy cattle (Gressley et al., 2011).

Upon ingestion, feed enters the reticulum where large feed particles are retained. These particles are ruminated, or regurgitated and re-masticated, thus decreasing particle size while increasing surface area and attachment sites for microorganisms (McAllister and Cheng, 1994). The reticulum and rumen are only partially separated by a fold of tissue; thus, these chambers are generally discussed together as the reticulo-rumen (Membrive, 2016). The rumen, rich in anaerobic microorganisms, is the primary site of microbial fermentation in cattle. The extent to which feed is degraded in the rumen is inversely related to rate of passage (ROP). Factors including particle size, dietary composition, and individual animal characteristics impact ROP, with smaller particle size, increased salivary production, and increased water consumption associated with increased ROP (Mir et al., 1991). Feed particles smaller than 1.18 mm exit the rumen through the reticular-omasal sphincter and enter the omasum (Membrive, 2016).

The role of the omasum is not fully understood. The luminal surface of the omasum is smoother than that of the reticulum and rumen. There are, however, multiple longitudinal folds which cause the surface area of the omasum to equal that of the rumen, though volumetric capacity of the omasum is $1/10^{th}$ that of the rumen (Membrive, 2016). The omasum absorbs water, peptides, and minerals, though less efficiently than the rumen (Engelhardt and Hauffe, 1975; Matthews and Webb, 1995; Xu et al., 2014). Omasal folds may retain feed particles and further reduce particle size through abrasive sliding of folds during peristalsis (Bost, 1981). Lastly, there is evidence of limited microbial activity in the omasum; however, the overall contribution of omasal fermentation is not well quantified (Smith, 1984).

Feed enters the abomasum through the omasal-abomasal sphincter. The abomasum is referred to as the true stomach, having glandular walls and secreting acid, similar to the stomach of a monogastric (Membrive, 2016). Hydrochloric acid reduces pH of digesta to less than 2.5,

inactivating microbial enzymes and activating abomasal enzymes, chiefly pepsin. Pepsin initiates digestion of protein, which will continue throughout the small intestine (Meitner and Kassell, 1971; Owens et al., 1986; Dijkstra et al., 2005). Availability of protein to the animal is affected by degradation of dietary protein in the rumen. Protein that is degraded in the rumen will be incorporated into the membranes and cell walls of microorganisms, commonly referred to as microbial cell protein (MCP). When microorganisms pass out of the rumen, MCP becomes available to the animal through digestion in the small intestine (Chalupa, 1974). Because amino acids – which can be used directly or synthesized by microbes from nitrogenous sources – are required for proliferation of ruminal microorganisms, dietary protein and non-protein nitrogen enhance microbial proliferation and thus fermentation (Hackmann and Firkins, 2015). Not surprisingly, there is evidence that feeding ruminal undegradable protein (RUP) leads to increased essential AA leaving the rumen compared to feeding similar amounts of rumen degradable protein (RDP; Ipharraguerre and Clark, 2005). Therefore, feeding RUP to cattle may be beneficial, as long as there is adequate nitrogen (N) available to support microbial activity in the rumen (Ipharraguerre and Clark, 2005).

In the small intestine pH is increased to approximately 7 and α-amylase released from the pancreas is mixed with digesta, marking the beginning of post-ruminal starch digestion (Owens et al., 1986). Starch entering the small intestine may have been unaltered or partially degraded by ruminal microorganisms, depending on dietary composition and ROP (Owens et al., 1986; Harmon, 2009). Starch digestion in the small intestine provides more energy to cattle than VFA produced through ruminal fermentation of starch; therefore, limiting ruminal degradation of starch is of interest (Owens et al., 1986). Hydrolyzation of dietary lipids within the rumen is generally greater than 85% (Bauman and Lock, 2006). Fatty acids and microbial phospholipids

leaving the rumen will remain relatively unaltered until entering the small intestine, which is the first site of absorption of fats and fatty acids in the ruminant. Absorption of free fatty acids is reliant upon the formation of micelles (Bauman and Lock, 2006). In cattle, formation of micelles is dependent upon secretion of bile from the gallbladder, which contains bile salts and lecithin, and pancreatic secretion of lipase (Lock et al., 2006; Gomez et al., 2017). The presence of taurine- and glycine-conjugate bile salts allows for the absorption of fatty acids in acidic conditions, enhancing absorption of fatty acids throughout the small intestine (Tamminga and Doreau, 1991; Bauman and Lock, 2006). Regardless of dietary component, the extent to which the small intestine digests and absorbs nutrients depends upon ruminal fermentation, gastric digestion, and ROP.

Digesta is subjected to fermentation throughout the large intestine where microorganisms degrade feed in a manner similar to what occurs in the rumen. Retention time of digesta in the large intestine of cattle, however, is approximately 9 h (Hecker and Grovum, 1975), whereas ruminal retention of digesta may be upwards of 72 h (Panjaitan et al., 2010). Due to reduced retention time, post-gastric fermentation of cellulose and hemicellulose may be limited. Another difference between ruminal and post-gastric fermentation is that MCP synthesized in the hindgut is excreted and therefore not available for utilization by the animal (Hristov et al., 2019). While the importance of fermentation in the large intestine is debatable, the large intestine serves as a final site of absorption for water, minerals, nutrients, and VFA (Bergman, 1990; McKie et al., 1991).

Ruminal fermentation

Carbohydrates

Plants contain large quantities of polysaccharides, which are chains of monosaccharides linked together through glycosidic bonds (Guo et al., 2017). The majority of energy requirements for ruminants are met through degradation of CHO into VFA in the rumen (Bergman, 1990; France and Dijkstra, 2005). There are 2 classifications of CHO: those associated with cell walls (structural; sCHO) and those associated with energy storage (nonstructural; nsCHO; Chesson and Forsberg, 1997). Structural CHO are degraded through microbial fermentation while nsCHO are degraded by both microbial and mammalian enzymes (Nozière et al., 2010). Chemical structure of monosaccharides, specifically presence of branches and types of bonds, affects microbial degradation of feed (Chesson and Forsberg, 1997).

Structural carbohydrates

Plant cell walls contain pectin, lignin, hemicellulose, and cellulose, and these components are collectively referred to as fiber (Chesson and Forsberg, 1997). Each component has a unique structure, necessitating a variety of microbial enzymes to degrade fiber (Coen and Dehority, 1970; Dai et al., 2015; Moraïs and Mizrahi, 2019). Fiber is a slowly fermented component of feed. Many cellulolytic microorganisms have complex protein structures known as carbohydrate-binding modules (CBM; Fig. 1.1) which aid in the fermentation of cellulose by attaching cellulolytic microorganisms directly to the surface of fiber (Chesson and Forsberg, 1997; Schwarz, 2001; Lynd et al., 2002; Moraïs and Mizrahi, 2019). As a result, cellulolytic enzymes are not freely secreted into ruminal fluid, thereby decreasing proteolysis of these enzymes. In addition to protecting cellulolytic enzymes, some CBM also break hydrogen bonds between chains of cellulose, serving as a catalyst for fermentation of cellulose (Miron et al., 2001; Krause

et al., 2003). The basic structure of a CBM includes a catalytic region which binds to substrate and amino acids that connect the catalytic region to the non-catalytic region near the bacterial cell surface (Schwarz, 2001). Within CBM are structures known as scaffoldins or cellulosomes. These multi-enzyme complexes become tightly associated with cellulose and aid in degradation of its crystalline structure (Chesson and Forsberg, 1997). The primary mode of action by which cellulosomes improve degradation is through non-specific binding on the feed molecule and synergistic enzymatic activity (Schwarz, 2001; Lynd et al., 2002). Fibrolytic microorganisms without CBM are reliant upon binding sites, which are generally restricted to cut edges of the fiber molecule. Therefore, degradation of fiber by some fibrolytic microorganisms is limited by particle size (Chesson and Forsberg, 1997; Scheller and Ulvskov, 2010).

While classified as a sCHO, pectin is part of the intracellular matrix and is comprised of D-galacturonic acid and side chains containing various sugars connected primarily through α-1,4 glycosidic bonds (Fig. 1.2; Van Buren, 1991; Căpriţă et al., 2010). Ruminal microorganisms produce pectin esterase, which rapidly degrades pectin into pectic acid and methanol. Microbial endo- and exo-polygalacturonases hydrolyze α-1,4 glycosidic bonds within pectic acid, yielding individual D-galacturonic acid molecules (Smart et al., 1964).

Lignin is comprised of 4-hydroxyphenylporpandoid polymers with multiple branch points and aromatic rings linked through carbon bonds (Fig. 1.3; de Gonzalo et al., 2016). Concentration of lignin in plants increases with maturity, contributing to the rigidity of upright stems while protecting against damage and water loss (Moore and Jung, 2001). Lignin is indigestible by both mammalian and microbial enzymes, with minor levels of solubilization occurring in the rumen through hydrolyzation of ether and ester linkages to other cell wall constituents (McSweeney et al., 1994; Raffrenato et al., 2017). Thus, as plant maturity increases,

there is a decrease in digestibility. Lignin cross-linkages also limit rate and extent to which cellulose and hemicellulose are degraded (Engels and Jung, 2005). Grabber et al. (2009) reported a 37% increase in lag time of gas production and decreased fermentation of hemicellulose *in vitro* when lignin content was artificially increased. There may be a similar response *in vivo* whereby increased lignin results in decreased degradation of hemicellulose, although this would be impacted by ROP. Raffrenato et al. (2017) found cross-linkages of lignin to hemicellulose to be more predicative of digestibility of neutral detergent fiber (NDF) in dairy cows than lignin content alone. Due to the important role of lignin within the plant (Rubin, 2008), genetic modification for decreased lignin content may be impractical; therefore, feeding immature plants with less lignin is a more realistic approach to enhancing fermentation of fiber.

Hemicellulose is a major component of cell walls in forages. The main chain is comprised of xylan molecules linked together via ß 1-4 glycosides, and it contains large quantities of side chains or branches (Fig. 1.4). The most common side chains contain arabinose or glucuronic acid, connected to the xylan chain through α-1,2 or α-1,3 linkages (Dehority, 1973; Cotta and Zeltwander, 1995; Ebringerová, 2006). Within the primary xylan chain, glycosidic bonds are separated by microbial endo- and exo-xylanases which hydrolyze random glycosidic bonds within the chain and the second-to-last glycosidic bond, respectively. Smaller chains are degraded by microbial xylanases while microbial xylobiase, xylobiose phosphorylase, and xylodextrin phosphorylase hydrolyze bonds between xylo-oligosaccharides and xylobiose (Hespell, 1988). The microbial enzymes necessary to liberate sides chains of hemicellulose will depend upon the sugar present at each branching point. Arabinofuranosidase yields arabinose and xylan, acetylxylan esterase yields acetyl groups and xlyan, and glucuronidase liberates methylgucuronate side chains, yielding glucoronate and xylan (Hespell and Whitehead, 1990).

Several ruminal microorganisms, including *Prevotella*, *Fibrobacter*, and *Ruminococcus* sp., produce enzymes to degrade bonds and structures found in hemicellulose (Avguštin et al., 1992; Dai et al., 2015; Moraïs and Mizrahi, 2019). Coen and Dehority (1970), however, reported most microorganisms that degrade hemicellulose do not utilize the pentose sugars yielded from fermentation of hemicellulose. Instead, other microorganisms are required for end-product utilization, demonstrating the importance of microbial cross-feeding (Coen and Dehority, 1970).

Cellulose comprises the greatest fraction of sCHO within forages. Concentration of cellulose is greater in the secondary cell wall, which forms once maturity of plants is reached (Jung, 1997; Căpriţă et al., 2010). Cellulose is a monomer of tightly packed glucose molecules rotated 180° and connected by β-1,4 glycosidic bonds (Fig. 1.5; Béguin and Aubert, 1994: Căpriţă et al., 2010; Moraïs and Mizrahi, 2019). The secondary structure of cellulose contains cross-linked glycan chains which form fibrils through hydrogen bonding (Chesson and Forsberg, 1997). These fibrils may be extremely ordered, forming a strong crystalline structure or less organized in amorphous regions. It is this secondary structure which contributes to insolubility of cellulose. Amorphous regions are more easily degraded, as water and cellulase enzymes may be unable to penetrate the crystalline structure (Chesson and Forsberg, 1997; Kerley et al., 1988). Weimer et al. (1991) reported greater rate of fermentation when amorphous cellulose was provided as substrate to both *R. flavefaciens* and *F. succinogenes*, while pure crystalline cellulose had the slowest rate of fermentation. Cellulose-fermenting bacteria within the rumen include *R. flavefaciens*, *F. succinogenes*, and *R. albus*, all of which contain CBM to adhere to cellulose (Chesson and Forsberg, 1997; Moraïs and Mizrahi, 2019). Cellulolytic enzymes produced by ruminal microorganisms include β-1,4 endo- and exo-glucanases that randomly hydrolyze bonds within the cellulose chain and the second-to-last glycosidic bond, respectively, yielding shorter

cellulose chains and cellulobiose. Microbial cellulodextrinase further reduces cellulodextrins, while β-1,4 glucosidase degrades remaining cellulobiose (Wang and McAllister, 2002).

One of the most influential factors impacting fermentation of fiber is ruminal pH. Bacterial pectic enzymes have optimal activity between pH 7 and 8, moderate activity with pH 6.2 to 6.8, and lose all activity if pH is < 5 (Smart et al., 1964). Meanwhile, xylanase activity for hemicellulose degradation is greatest when pH is between 6 and 7, with a rapid decrease in activity when pH < 6 (Morrison, 1976; Lowe et al., 1987). Cellulose degradation is also affected by pH, as the ability of cellulolytic bacteria to adhere to fiber is pH dependent. Sung et al. (2006) reported greater unattached populations of *F succinogenes*, *R. flavefaciens*, and *R. albus* with pH 5.7 compared to 6.2 and 6.7, with a subsequent reduction in degradation of fiber. Hu et al. (2004) reported optimum ruminal pH of 6.8 for degradation of cellulose with reduced efficiency when pH was increased or decreased. Thus, maintaining ruminal pH > 6 is imperative for fermentation of fiber.

Non-structural carbohydrates

Simple sugars, short fructooligosaccharides, and starch are classified as nsCHO. Starch is used to store energy in the intracellular matrix of plants and has a complex crystalline structure comprised of amylose and amylopectin (Tester et al., 2004). Amylose is a straight-chained homopolymer of glucose connected via α-1,4 glycosidic bonds (Fig. 1.6; Manners, 1989). Amylopectin is also a homopolymer of glucose connected via α-1,4 glycosidic bonds, but it contains side chains connected to the main glucose chain via α-1,6 glycosidic bonds (Fig. 1.7; French, 1972; Manners, 1989). In the rumen, amylolytic microorganisms release amylases that cleave α-1,4 glycosidic bonds. Alpha amylases randomly cleave α-1,4 glycosidic bonds, and terminal maltose molecules are targeted by β-amylases. Microbial maltases cleave maltose into 2

glucose molecules (Santos et al., 2016). Amylopectins require a unique enzyme, pullulanase, to cleave α-1,6 glycosidic bonds (French, 1972; Rooney and Pflugfelder, 1986). Several microorganisms are considered amylolytic and have the capability to degrade starch, sugars, or both. Some of the more prominent amylolytic microorganisms in the rumen are *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola, Succinimonas amylolytica, Selenomonas ruminantium, Butyrivibrio fibrisolvens, Eubacterium ruminantium*, and *Clostridium* sp. (Cotta, 1988; Chesson and Forsberg, 1997). The same bacteria also degrade amylopectin, with bacteria within the *Ruminobacter, Streptococcus*, and *Bifidobacterium* genera producing pullulanase to remove α-1,6 bonds (Chesson and Forsberg, 1997; Flint et al., 2012).

Quantity of nsCHO in plants varies throughout the day, being greater in the afternoon following photosynthesis and less in the morning following utilization of stored energy during the night (Owens et al., 2002; Bélanger et al., 2010). Non-structural CHO content is concentrated and less variable in seeds (Pfister and Zeeman, 2016); thus, grains are utilized to increase energy density of diets and increase feed efficiency. Type of grain and processing technique impact ruminal degradation of starch and, consequently, animal production (Stock et al., 1987; Huntington, 1997; Das et al., 2015). Stock et al. (1987) reported greater gain in animals supplemented with a mix of rapidly and slowly fermented grains versus those provided either type of grain alone.

Sudden inclusion of large quantities of nsCHO or feeding large quantities of nsCHO for extended periods may negatively impact ruminal conditions and be detrimental to the animal (Danscher et al., 2011). Amylolytic microorganisms rapidly degrade nsCHO into VFA and other organic acids, such as lactic acid (Danscher et al., 2011). When rate of acid production exceeds rate of absorption and bacterial utilization, accumulation of acid occurs, thus reducing ruminal

pH. Under normal feeding conditions ruminal pH is maintained between 5.6 and 6.8 with reductions often following feeding (Nagaraja and Titgemeyer, 2007). When feeding large quantities of starch for extended periods, however, pH may be maintained between 5.5 and 5.0, resulting in subacute ruminal acidosis (Nagaraja and Titgemeyer, 2007). While populations of fibrolytic bacteria persist when ruminal pH is between 5.5 and 5.0, there is a slight reduction in enzymatic activity (Kitts and Underkofler, 1954; Erfle et al., 1982). As pH declines further, many cellulolytic bacteria may be inhibited or eliminated, resulting in depressed degradation of fiber (Nagaraja and Titgemeyer, 2007). If bacterial death occurs, endotoxins will be released, resulting in further bacterial death and additional release of endotoxins (Li et al., 2012). Leaching of endotoxins into the bloodstream may lead to laminitis, depressed feed intake, further reduction in degradation and utilization of feed, and potentially death (Nagaraja et al., 1978a; Nagaraja et al., 1978b; Suda et al., 1994).

Lipids

Level and composition of lipids in ruminant diets vary greatly depending upon ingredients. Lipid content of forage-only diets consists primarily of glycolipids and phospholipids found within plant membranes and ranges between 4 and 8% (Harfoot and Hazlewood, 1997; Arrigoni et al., 2016). Cattle fed concentrates may have much greater percentages of dietary lipids, as seeds can contain as much as 50% triglycerides (Arrigoni et al., 2016). Supplemental oils and fats, such as soybean oil and linseed oil, may also be included in rations to increase energy density.

Microbial lipases immediately hydrolyze ester bonds, liberating fatty acids from the alcohol backbone (Jenkins, 1993). Unsaturated free fatty acids are then subjected to biohydrogenation, a process by which isomerization may occur and double bonds between

carbons are removed through the addition of hydrogen (Buccioni et al., 2012). Biohydrogenation is believed to protect ruminal microorganisms, as unsaturated fatty acids can interrupt the cellular membrane of ruminal microorganisms, altering intramembranous pathways and leading to ionic imbalance (Maia et al., 2010). Maczulak et al. (1981) reported inhibitory effects of 2 unsaturated fatty acids (oleic and vaccenic acids) on cellulolytic bacteria. Likely due to this inhibitory effect, there is a reduction in degradation of fiber when dietary fats exceed 6% (Salem et al., 1993; Broudiscou et al., 1994). In addition to inhibiting cellulolytic bacteria, dietary fats may further reduce fermentation of fiber by coating feed particles and preventing fibrolytic enzymes from adhering to the surface of sCHO (Jenkins, 1993; Hess et al., 2008).

Protein

The recommended dietary crude protein (CP) content for forage-fed cattle is 7% to 11%, a level that avoids reduction in dry matter intake (DMI) while optimizing digestibility (Moore and Kunkle, 1995; Köster et al., 1996). Köster et al. (1996) reported feeding > 11% CP led to reduced DMI, which is not optimal because cattle who consume more feed generally produce greater quantities of milk or gain more weight than cattle who consume less feed. Protein content in forages varies, ranging from 4% in poor quality warm-season grasses to 20% in legumes, but not all protein is degradable by ruminal microorganisms (Jurgens et al., 2012). Rumen degradable protein serves as a source of N that is utilized by microorganisms for cellular maintenance and proliferation (Oh et al., 2008). It has long been established RDP is the first limiting nutrient for ruminants fed low-quality forage (Köster et al., 1996). Protein solubility is often utilized as the primary indicator of RDP; however, protein structure is also an important consideration as microorganisms have limited ability to degrade sulfide bonds (Mahadevan et al., 1980).

Ruminal microorganisms release proteases and peptidases to degrade peptide bonds in proteins and peptides, respectively, yielding smaller units until short amino acid chains or individual amino acids are liberated (Van Straalen and Tamminga, 1990). Most amino acids are deaminated into ammonia and transported into microbial cells for cellular maintenance, repair, and proliferation (Griswold et al., 1996; Hristov et al., 2004; Oh et al., 2008). Thus, urea, which is rapidly converted to ammonia in the rumen, serves as a cost-effective non-protein nitrogen (NPN) source to meet microbial N requirements (Griswold et al., 2003). Fibrolytic bacteria, however, require branch-chained amino acids for growth (Stewart et al., 1997). Therefore, to optimize fermentation of fiber, protein sources rich in branched-chain amino acids such as sodium caseinate may be provided (Griswold et al., 1996; Köster et al., 1996).

Another important consideration when supplementing protein is to balance RDP with available CHO, as ruminal microorganisms require both N and energy to proliferate. Over-supplementation of N without adequate CHO leads to ammonia accumulation within the rumen, which will be absorbed into the blood stream and excreted from the animal (Hackmann and Firkins, 2015). Inadequate RDP, however, leads to energy imbalance in the rumen. Degradation of CHO via ruminal microorganisms yields VFA. These VFA can be utilized by microorganisms as a source of energy for cellular repair and proliferation. When, however, greater amounts of energy are present than what can be utilized for microbial growth, the energy is either converted into glycogen and stored intracellularly in microorganisms, or energy spilling occurs, whereby energy is released via production of heat (Hackmann and Firkins, 2015). Both energy storing and energy spilling are wasteful actions. Therefore, synchronized availability of N and energy is important to maximize microbial growth and efficiency. Amino acids provided to the animal through RUP are also important for improvement of animal production, as RUP is unaltered by

microbial fermentation and offers a greater variety of essential AA than MCP (Ipharraguerre and Clark, 2005). Thus, a balance between RDP and RUP should be provided to maximize utilization of dietary forages and animal production.

Supplements to enhance fermentation of fiber

Ionophores

Ionophores are large, lipophilic molecules that are absorbed into the membrane of gram-positive bacteria and transport cations across the membrane (Russel and Strobel, 1989; McGuffey et al., 2001). These bacteria must then use additional energy to maintain concentration gradients, limiting energy available for growth and proliferation (Painter et al., 1982). Thus, affected microorganisms are either inactivated, but remain in the rumen, or they are out-competed for nutrients, leading to cellular death and removal from the rumen (Russel and Strobel, 1989; Azzaz et al., 2015). Because gram-negative bacteria have 2 lipid membranes (Russel and Strobel, 1989) and ionophores only interact with the outer membrane, these bacteria are protected from effects of ionophores (Azzaz et al., 2015).

Nagaraja et al. (1982) reported intraruminal administration of ionophore antibiotics (Tylosin and Monensin) in cattle reduced populations of *S. Bovis* and *Lactobacilli,* which are starch degrading microorganisms that contribute to rapid accumulation of lactate and subsequent decreases in ruminal pH (Bryant, 1959; Cotta, 1988; Nagaraja, 2016). Therefore, selection against gram-positive bacteria helps protect against lactic acidosis and subsequent reductions in degradation of fiber. In healthy cattle consuming forage diets, gram-positive bacteria account for approximately 10.7% of ruminal bacteria. However, 9 h following induction of lactic acidosis, populations of gram-positive bacteria increase to 45.6% (Nagaraja et al., 1978b). Therefore, ionophores target a small population of ruminal bacteria in healthy animals and mitigate the

rapid proliferation of gram-positive bacteria when animals consume large quantities of starch. The complete removal of gram-positive bacteria through supplementation of ionophores is unlikely, decreasing the possibility of negative side-effects (Nagaraja et al., 1982). Reducing the relative abundance of gram-positive bacteria also decreases competition for protein, VFA, and carbon, thus increasing nutrient availability for fibrolytic bacteria. Lastly, ionophores reduce proteolysis in the rumen and decrease methane production by 26% (Wedegaertner and Johnson, 1983). Protein that escapes ruminal degradation is available to the animal and carbon, which would normally be converted to methane during ruminal fermentation, is transformed into propionate, a gluconeogenetic compound (Wallace et al., 1980; Nagaraja, 1995).

Buffering agents

Because cellulolytic and hemicellulolytic enzymes have an optimal pH of approximately 6.8, maintaining ruminal pH $\geq$ 6 is important for maximizing ruminal fermentation of sCHO (Morrison, 1976; Lowe et al., 1987; Hu et al., 2004). Buffering agents such as carbonate, bicarbonate, potassium, and magnesium attenuate post-prandial decreases in ruminal pH, which may prevent subsequent reductions in fermentation of fiber (Erdman, 1988). While buffering capacity refers to the minimal amount of a strong acid or base needed to change the pH of 1 L of solution by 1 unit (Karow et al., 2013), this measurement may become confounded as VFA are released during fermentation. Therefore, buffering value index (BVI) may be more accurate as it accounts for ruminal acidity and buffering capacity (Tucker et al., 1992). Le Ruyet and Tucker (1992) evaluated 4 buffering agents and reported increased ruminal pH and BVI with all 4 buffers; however, activation time varied with product. Thus, it is important to consider when the buffering agent will be most needed. In some cases, supplementation of both fast and slow activating buffers may be required to optimize ruminal conditions for fermentation. Rogers et al.

(1982) reported greater ruminal pH; milk production; digestibility of dry matter (DM), organic matter (OM), and starch; and a tendency for increased digestibility of acid detergent fiber (ADF) when feeding dairy cows a 75% concentrate diet supplemented with 2% sodium bicarbonate compared to a negative control. Kennelly et al. (1999), however, reported supplementing a 75% concentrate diet with 1.2% sodium bicarbonate had no impact on total tract digestibility of DM, NDF, or ADF. Therefore, it appears ruminal fermentation of diets containing 75% concentrate may only benefit from sodium bicarbonate if supplied at a rate ≥ 2%.

Direct-fed microbials

The terms probiotic and direct-fed microbials (DFM) are frequently used interchangeably, though they refer to 2 different types of supplements. Probiotics are classified by the Food and Agriculture Organization/World Health Organization (2002) as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host." The term probiotic, however, has also been used to describe culture extracts, microbial enzymes, and combinations of all 3 (Yoon and Stern, 1995). Due to potential confusion, the Food and Drug Administration requires a more specific term, DFM, be used to describe products containing live cells native to the rumen (Elghandour et al., 2015). Direct-fed microbials have the potential to colonize the rumen, offering prolonged benefits (Krehbiel et al., 2003). There are 3 primary categories of DFM: bacteria, fungi, or a combination of both (Seo et al., 2010).

Two of the most commonly utilized bacterial types in DFM include lactic acid producing bacteria (LAB) and lactic acid utilizing bacteria (LUB). Products with lactic acid producing bacteria frequently contain *Enterococcus, Streptococcus, Lactobacillus,* or *Pediococcus* (Brink et al., 2006; McAllister et al., 2011). Nocek et al. (2003) reported cows supplemented with *Enterococcus sp.* had increased DMI, milk production, and milk protein percentage during the

first 21 d of lactation. Supplementing LAB may acclimate ruminal microorganisms to the presence of lactic acid and stimulate growth of LUB, thus preventing or reducing severity of acidosis (Seo et al., 2010; McAllister et al., 2011). Supporting this theory, Qadis et al. (2014) reported supplementation of LAB resulted in more consistent ruminal pH and stable microbial populations when feeding Holstein calves 50% concentrate diets. Furthermore, LAB are antimicrobial and may lead to competitive exclusion of pathogenic microorganisms, such as *E. coli*, while stimulating the immune system of the host (Brink et al., 2006; Tabe et al., 2008; Dicks and Botes, 2010).

Lactic acid utilizing bacteria may be supplemented to increase fermentation of lactate, thus reducing accumulation of lactate and subsequent ruminal acidosis (Seo et al., 2010; McAllister et al., 2011). The 2 predominant LUB in the rumen are *Megasphaera elsdenii* and *Propionibacterium*, both of which yield propionate as an end product (Marounek et al., 1989; Seo et al., 2010). Kung and Hession (1995) found inoculating ruminal cultures with *Megasphaera elsdenii* reduced lactate and increased VFA concentrations, indicating improved fermentation. Stein et al. (2006) reported supplementing *Propionibacterium* to dairy cows increased ruminal concentration of propionate and increased milk production.

Supplementation with fungal DFM, primarily *Aspergillus sp.* and *Saccharomyces cerevisiae*, has been extensively researched in ruminants (Yoon and Stern, 1995). Gomez-Alarcon et al. (1990) reported supplementation with *Aspergillus oryzae* led to increased ruminal and total tract digestibility of fiber, even though ruminal VFA were not affected. Supplementation with *Saccharomyces cerevisiae* has been shown to increase ruminal pH, ruminal VFA concentrations, total-tract digestibility of OM, DMI, and milk yield, while decreasing lactic acid concentration in the rumen (Tricarico et al., 2008; Desnoyers et al., 2009).

Conversely, Chiquette (1995) reported no difference in DMI, total tract digestibility, milk yield, or milk composition when supplementing dairy cattle with *Aspergillus oryzae*, *Saccharomyces cerevisiae*, or both when compared to control.

Exogenous enzymes

Another feed additive of microbial origin used to enhance ruminal fermentation is EE. Native ruminal microorganisms are selected for their production of targeted enzymes and grown in a laboratory setting (Beauchemin et al., 2003). After approximately 7 d, media is collected and enzymes separated for preparation of concentrated EE products (Lee et al., 1998; Abrão et al., 2017).

There are 4 classifications of EE: proteolytic, saccharolytic, lipolytic, and fibrolytic (Beauchemin et al., 2004a). Exogenous fibrolytic enzymes are most utilized as they enhance ruminal degradation of sCHO, which are slowly fermented in the rumen. Microorganisms most frequently cultured to produce EFE include fungi (*Trichoderma longibrachiatum*, *Trichoderma reesei*, *Aspergillus niger*, *Aspergillus oryzae*) and bacteria (*Bacillus*), with each source producing varying amounts of cellulases and xylanases to degrade cellulose and hemicellulose, respectively (McAllister et al., 2001; Wang and McAllister, 2002). In addition, other enzymes such as pectinases and glucanases are often mixed into EFE to aid in degradation of liberated compounds.

Exogenous fibrolytic enzymes

Measuring enzymatic activity

There are multiple methods utilized to measure enzymatic activity of EFE, including the spectrophotometric method, the carboxymethyl cellulase (CMCase) activity method, and filter

paper analysis (Shuangqi et al., 2011). Specific substrate, incubation time, pH, and temperature will vary depending upon the assay and targeted enzyme.

The spectrophotometric method can be used to evaluate a wide range of enzymes; thus, there is much variation in substrate utilized. Other components of this assay include a colorimetric additive attached to the substrate and buffering agents. As substrate is degraded, there is a corresponding change in color of the solution, representing activity of the enzyme. A spectrophotometer with wavelength set to approximately 500 nm is used to measure the change in color based upon light absorbance (Hayakari et al., 1978; Shuangqi et al., 2011). This assay is relatively quick, taking between 1 and 60 min to complete depending on protocol and can be used to measure enzymatic activity throughout the duration of the reaction (Hayakari et al., 1978; Aich et al., 2001; Hadwan, 2018). While the spectrophotometric assay was one of the first analyses to produce fast and consistent results, substrates rich in chlorophyll or phenolic compounds may lead to background interference and impact the color being analyzed (Shuangqi et al., 2011; Johnsen and Krause, 2014). Chlorophyll and phenolic compounds absorb light and, as a result, spectrophotometers may need to be set to alternate wavelengths in order to get accurate readings (Haskin, 1942; Dearden and Forbes, 1959).

The CMCase activity assay, sometimes referred to as the carboxymethylcellulose (CMC) method, is utilized to measure cellulase activity using culture media containing carboxymethylcellulose (Dashtban et al., 2010). Enzymes are added to culture media in well plates and allowed to incubate for a pre-determined length of time prior to centrifugation, which suspends reducing sugars in the supernatant (Abrão et al., 2017). The amount of reducing sugars present can then be quantified with dinitrosalicylic acid to determine enzymatic activity (Miller, 1959). Another version of this assay requires measuring viscosity of media via a viscometer. As

cellulose is degraded, the viscosity of media is reduced (Shuangqi et al., 2011). Carboxymethyl cellulase activity assays are frequently utilized in industry as multiple samples can be analyzed in tandem (Shuangqi et al., 2011). Reproducibility has been problematic though, with CMC source affecting concentration of reducing sugars (Dashtban et al., 2010). Analyses of xylanase activity can be performed in a similar manner, with substitution of birchwood or beechwood glucuronoxylan, oat spelt arabinoxylan, DMSO acetyl glucuronoxylan, or insoluble, unsubstituted beechwood xylan for CMC (Bailey et al., 1992; Houfani et al., 2019).

The filter paper assay measures total cellulase activity (Dashtban et al., 2010). Historically, enzymes were added to Whatman no. 1 filter paper and time required for total collapse of filter paper was recorded to determine enzyme activity. Time until collapse, however, is inconsistent, resulting in large error rates (Shuangqi et al., 2011). A more standardized methodology for this assay, designed to reduce error, is measuring the amount of glucose liberated after 1 minute of incubation (Dashtban et al., 2010).

Effects of exogenous fibrolytic enzymes on nutrient digestibility

Application of EFE to ruminant diets was common in the 1960's, with evidence of improved weight gain and feed efficiency in feedlot steers (Burroughs et al., 1960). Enzymes used and amount applied varied in early published literature. In addition, diets to which enzyme products were applied also differed. As a result, responses to EFE inclusion in ruminant diets were inconsistent, leading to the theory enzyme proteins were rapidly degraded by ruminal microorganisms (Leatherwood et al., 1960; Templeton and Dyer, 1967; Beauchemin et al., 2003). This theory, however, was disproven as 2 EFE products remained active for 15 h within the rumen (Hristov et al., 1998a). This revelation led to renewed interest in EFE, with emphasis on application rates, enzyme ratios, and application method.

Researchers evaluating the effect of EFE produced from *Trichoderma* have reported increased dry matter digestibility (DMD) when feeding enzyme-treated total mixed rations (TMR) to steers (Beauchemin et al., 1995) and lactating cows (Lewis et al., 1999; El-Bordeny et al., 2015); however, this is not always observed (Dhiman et al., 2002). Perhaps more important than DMD is digestibility of fiber. The inclusion of EFE has been shown to increase fermentation of NDF and ADF (Beauchemin, et al., 1999; López-Aguirre et al., 2016). López-Aguirre et al. (2016) reported increased digestibility of NDF when lambs were fed enzymes produced from *Trichoderma longibrachiatum*. Pinos-Rodríguez et al. (2008), however, reported that while enzymes produced by *Trichoderma longibrachiatum* and *Aspergillus niger* led to increased *in vitro* disappearance of NDF, the same effect was not observed *in vivo* when lambs were fed enzyme-treated TMR. Beauchemin et al. (1999) reported feeding enzymes produced from *Trichoderma longibrachiatum* to dairy cattle had no impact on total tract digestibility, but ruminal digestibility of NDF was greater. Conversely, total tract digestibility of ADF was greater when dairy cattle consumed TMR treated with enzymes produced from *Trichoderma longibrachiatum*, while ruminal digestibility of ADF was unaffected (Beauchemin et al., 1999). Yang et al. (2000), however, reported no effect of enzymes produced by *Trichoderma longibrachiatum* on total tract digestibility of ADF when fed to dairy cattle.

Others have reported that dairy cattle consuming enzymes produced by *Trichoderma longibrachiatum* supplied via TMR or concentrate had increased total tract digestibility of CP, ADF (Beauchemin et al., 1999), and OM (Yang et al., 2000). Interestingly, digestibility of N in the rumen was unaltered (Beauchemin et al., 1999). Therefore, it has been theorized that greater fermentation of fiber while N is available in the rumen may increase microbial proliferation and, consequently, increase MCP entering the small intestine through normal removal of

microorganisms from the rumen (Beauchemin et al., 1999). Having greater quantities of MCP in the small intestine, while maintaining ruminal fermentation of N, would increase the amount of CP degraded throughout the entire gastrointestinal tract. However, due to the required synchronicity of availability of N and CHO for maximum production of MCP, supplementation of EFE is likely to increase total tract digestibility of N only when digestibility of other feed components such as DM or NDF and ADF are increased. This theory is supported by Silva et al. (2016) who reported no impact on total tract digestibility of CP, DM, or NDF when feeding TMR treated with enzymes produced by *Trichoderma longibrachiatum* to dairy cattle.

Effects of exogenous fibrolytic enzymes on animal performance

As expected with changes in nutrient digestibility, inclusion of EFE may alter animal performance. Exogenous fibrolytic enzymes are more often applied to dairy cattle rations; therefore, milk production and composition are commonly evaluated to determine effect of EFE. Milk production is increased when EFE are applied to TMR (Lewis et al., 1999; Kung et al., 2000; Zheng et al., 2000) and concentrates (Bowman et al., 2002; Yang et al., 2000) in rations for dairy cattle. Applying enzymes produced by *Trichoderma longibrachiatum* to forages prior to mixing into TMR increases percentage of fat and protein in milk, indicating more complete utilization of feed (Schingoethe et al., 1999; Kung et al., 2000). Occasionally, EFE have no impact on milk production (Beauchemin et al., 2000) which has been attributed to over supplementation of EFE, resulting in excessive release of phenolic compounds which are toxic to some ruminal microorganisms (Lewis et al., 1999; Kung et al., 2000).

Feed efficiency or feed conversion is a ratio of feed intake relative to weight gained, with lower ratios indicating greater efficiency (McAllister et al., 1999). The effect of EFE on feed efficiency is variable. Balci et al. (2007) applied enzymes produced from *Trichoderma*

longibrachiatum to the concentrate portion of a growing steer ration and reported improved feed conversion ratio (FCR) of both the total ration and concentrate, but not the straw portion of the diet. Because EFE are supposed to enhance digestibility of fiber, the lack of difference reported regarding FCR of straw was unexpected and may be a result of EFE application to the concentrate as opposed to the forage. Thus, efficacy of EFE is likely impacted by dietary component to which they are applied. McAllister et al. (1999) reported an improved FCR when EFE were applied to the silage portion of a TMR and fed to growing steers from days 0 to 56, but not days 56 to 120. The reason EFE did not impact FCR as the experiment progressed is unknown; however, the steers had been purchased from an auction prior to the experiment and therefore may have been nutritionally challenged. Supplying EFE may have aided in stabilizing ruminal conditions in these animals, resulting in greater feed efficiency during the first portion of the trial. In another experiment by McAllister et al. (1999), supplying finishing steers with EFE sprayed onto the entire TMR did not impact FCR. The authors theorized differences in the effect of EFE on FCR between the growing and finishing steer trials were likely due to differences in diet.

Dry matter intake and average daily gain (ADG) are the 2 contributing factors which determine FCR and are important considerations when feeding EFE. There are several reports of increased DMI when feeding EFE produced from *Trichoderma sp.* (Beauchemin et al., 1995; Beauchemin et al., 2000; Silva et al., 2016) and EFE produced by mixed anaerobic ruminal bacteria (Gado et al., 2009). Others, however, have reported no impact on DMI when supplementing EFE from *Trichoderma sp.* (Beauchemin et al., 1997; Beauchemin et al., 1999; Rode et al., 1999; Yang et al., 2000). Generally, EFE applied to forage or entire TMR leads to increased DMI, while application of EFE to concentration portions of TMR has no effect.

Increased DMI when EFE are applied to the forage portion of a TMR or the entire TMR may be due to pre-ingestive hydrolysis of fiber, which increases the quantity of reducing sugars. As a result, feed may taste sweeter, stimulating intake. Therefore, application method of EFE likely impacts the effect of EFE on DMI. Increasing DMI is generally a goal of producers as cattle that consume greater quantities of feed are likely to have greater ADG, reducing the amount of time required to reach market condition. Beauchemin et al. (2000) found applying EFE produced by *Trichoderma longibrachiatum* to TMR increased ADG, while Zobell et al. (2000) reported only a tendency for increased ADG. Alternatively, others have reported no changes in ADG with supplementation of EFE (Beauchemin et al., 1997; Beauchemin et al., 2000; Kung et al., 2000). Impact of EFE on ADG is likely dependent upon dietary formulation. Cattle consuming alfalfa hay treated with EFE had increased ADG, but cattle consuming barley silage treated with EFE did not (Beauchemin et al., 1995).

Method and rate of application

Ruminal fermentation, total tract digestibility of nutrients, and animal performance may increase when feeding EFE, though it is difficult to compare data obtained between experiments due to variation in source, amount, and application method of enzymes, as well as composition of animal diets. Application of EFE onto forages or TMR is commonly performed by diluting liquid enzyme or dissolving granular enzyme product in water to increase volume to allow consistent distribution throughout the feed. There have been multiple reports of increased digestibility of DM, NDF, and ADF (Beauchemin et al., 1995; Beauchemin et al., 1999; Lewis et al., 1999; El-Bordeny et al., 2015; López-Aquirre et al., 2016) and increased milk production (Lewis et al., 1999; Kung et al., 2000; Zheng et al., 2000), DMI (Lewis et al., 1999; Beauchemin et al., 2000; Kung et al., 2000), and ADG (Beauchemin et al., 1995) when EFE are sprayed on

TMR or forages. Application in this manner is thought to be superior, as EFE are in direct contact with the largest portion of the diet.

Addition of EFE to concentrate or premix portions of TMR has also been extensively evaluated, though there are challenges associated with pelleting active enzymes and potential sorting of diets. Application of EFE to concentrates has led to increased digestibility of DM, OM (Beauchemin et al., 2000; Yang et al., 2000; Bowman et al., 2002), and ADF (Krause et al., 1998). Meanwhile, Silva et al. (2016), Knowlton et al. (2007), and Krause et al. (1998) reported no effect of EFE on digestibility of DM and Beauchemin et al. (2000) and Yang et al. (2000) found no effect on fiber degradation when EFE were applied to concentrates. Beneficial effects of EFE are more likely when EFE are applied to greater proportions of the diet (Bowman et al., 2002; Beauchemin et al., 2004a; Beauchemin et al., 2004b); therefore, mixed results regarding degradation of fiber when EFE are applied to concentrates may be due to limited contact of EFE with the ration, specifically the forage. Another theory regarding limited efficacy of EFE in enhancing degradation of fiber when applied to concentrate portions of rations is that there is limited availability of moisture. Hydrolysis of fiber is dependent upon moisture, with silage components of rations containing greater quantities of moisture than concentrates (Beauchemin et al., 2004a). As a result, application of EFE to concentrates, particularly when a dried, granular EFE product is utilized, may not provide adequate moisture to allow maximal pre-ingestive hydrolysis of fiber (Beauchemin et al., 2004a). Important considerations concerning feed digestibility and utilization when applying EFE to concentrates also include whether enzymes were applied prior to manufacturing, whether heat may have denatured enzyme proteins, and length of storage.

The amount of enzyme applied also impacts the effect of supplementation of EFE on animal performance and nutrient digestibility. Lewis et al. (1999) fed EFE produced from *Trichoderma reesei* in a barley and alfalfa silage ration to dairy cows. Applying 1.25, 2.5, or 5.0 mL enzyme/kg forage (DM) increased DMI and decreased milk protein, but only the 2.5 mL/kg forage (DM) application rate led to increased total milk production (Lewis et al., 1999). Beauchemin et al. (1995) evaluated 5 enzyme application rates on different rations and reported greater ADG, DMI, and digestible DMI when 4,733 IU xylanase/kg (DM) were applied to alfalfa. Timothy hay required 12,000 IU xylanase/kg (DM) to maximize ADG, DMI, and ADF digestibility (Beauchemin et al., 1995). Kung et al. (2000) reported application of 3,500 CMCase units/kg forage (DM) and 16,000 xylanase units/kg forage (DM) resulted in increased milk yield and fat corrected milk/DMI compared to application of 8,800 CMCase units/kg forage (DM) and 40,000 xylanase units/kg forage (DM). It is possible over-supplementation of EFE liberates too many phenolic compounds while hydrolyzing cross-linkages between lignin and hemicellulose. While some phenolic compounds are hydrogenated within the rumen thus neutralizing toxic effects, other phenolics are not (Chesson et al., 1982). This may reduce bacterial populations, accounting for the lack of effect when EFE are supplemented at greater concentrations (Wang and McAllister, 2002).

Dietary composition

Two common grains included in dairy and beef cattle rations are corn and barley, and the type of grain included in TMR may affect efficacy of EFE. It should be noted, however, that application method confounds dietary composition, as application of EFE to concentrates is less likely to affect nutrient digestibility and animal performance than application of EFE to forages or total TMR. Researchers have reported increased milk production and digestibility of DM and

OM when the primary grain in the TMR was barley (Rode et al., 1999; Beauchemin et al., 2000; Yang et al., 2000; Bowman et al., 2002). Meanwhile, EFE applied to corn-based concentrates prior to mixing the concentrate into the forage component of the TMR had no impact on these same parameters (Knowlton et al., 2007; Silva et al., 2016). Increasing concentrate to > 90% of the ration may reduce the impact of EFE supplementation due to the reduction in dietary fiber. Beauchemin et al. (1997) reported addition of EFE to either barley or corn-based concentrate included at approximately 91% of the ration had no impact on ADG of steers, though FCR for the barley-based ration was improved through EFE supplementation. Krause et al. (1998) found EFE applied to a 95% barley-based concentrate ration had no impact on total DMD in growing steers, while ADF digestibility was increased. The greater fiber proportion in barley compared to corn is likely the reason EFE is more beneficial when applied to barley-based concentrates (Jurgens et al., 2012). The effect of grain source is less clear when EFE are applied to only the forage components of TMR (Schingoethe et al., 1999), or to the total TMR (Wiedmeier et al., 1987; Gomez-Alarcon et al., 1990; Beauchemin et al., 1999), as cattle consuming rations containing various grains, including barley, corn, and grain sorghum, have increased nutrient digestibility and performance.

Proposed mode of action

There have been several modes of action proposed concerning EFE. Pre-ingestive hydrolyzation of fiber is commonly reported as the primary mode of action (McAllister et al., 2001). Supporting this, release of reducing sugars, a product of hydrolyzation of glycosidic bonds, has been reported following EFE application to TMR (Hristov et al., 1996; Hristov et al., 1998a; Wang et al., 2001). This leads to reduced NDF content of forages prior to ingestion (Gwayumba and Christensen, 1997; Hristov et al., 1998a; Krause et al., 1998). Pre-ingestive

degradation of NDF removes barriers that limit microbial attachment and fermentation in the rumen; thus, fermentation rate of fiber may increase with EFE supplementation, particularly when ROP through the rumen is rapid (Beauchemin et al., 2004b). Enzymes produced from *Trichoderma longibrachiatum* increased *in vitro* attachment of *F. succinogenes* to corn silage and alfalfa when supplemented in moderate amounts; however, it decreased attachment of *F. succinogenes* to pure cellulose (Morgavi et al., 2004). The authors theorized that cellulose binding proteins of *Trichoderma longibrachiatum* may have out-competed CBM of *F. succinogenes* for binding sites on cellulose, preventing adhesion of *F. succinogenes* to cellulose (Morgavi et al., 2004). The outer structure of corn silage and alfalfa are more complex, containing different proteins and carbohydrates than cellulose; therefore, addition of *Trichoderma longibrachiatum* likely did not limit attachment sites for *F. succinogenes* when applied to cultures containing mixed substrate (Morgavi et al., 2004).

There also may be ruminal effects of EFE whereby enzymes aid in degradation of feed within the rumen by working synergistically with ruminal microorganisms, thereby increasing rate of fermentation. Morgavi et al. (2000) demonstrated a synergistic relationship between EFE and native ruminal microorganisms when introducing enzymes produced by *Trichoderma longibrachiatum* to ruminal cultures fed corn silage. This may be due in part to the ability of EFE to function at lower optimal pH than ruminal microorganisms, 4.5 to 5.0 vs. 6.2 to 6.8, respectively. However, the ability of EFE to enhance ruminal fermentation through enzymatic activity in the rumen itself has been questioned due to the large quantity of enzymes already present in the rumen (McAllister et al., 2001).

Altered proportions of ruminal microorganisms may contribute to increased fermentation of fiber when feeding EFE. Application of *Trichoderma longibrachiatum* enzymes to barley and

alfalfa fed to bacterial cultures led to increased cellulolytic species (Wang et al., 2001). Alternatively, Nsereko et al. (2002) applied *Trichoderma longibrachiatum* enzymes to a barley, alfalfa, and corn silage TMR and found no changes in cellulolytic populations, while total cellobiose-utilizing, xylanolytic and amylolytic bacterial populations increased *in vitro*. When evaluating relative proportions, however, only populations of cellobiose-utilizing microorganisms were increased (Nsereko et al., 2002). Differences observed in microbial populations may be due to application rates of EFE, composition of feed, or varying proportions of cellulases to xylanases between EFE products. Xylanases and cellulases liberate different intermediate products of fermentation; therefore, supplying these enzymes in different proportions and rates will likely stimulate proliferation of different ruminal microorganisms. It should be mentioned, however, the ability to identify ruminal microorganisms was extremely limited in the early 2000's, relying on statistical approaches (most probable number; Wang et al., 2001) or culturing techniques. As a result, reported effects on microbial populations were restricted to functional groups, not specific microorganisms. It should also be noted that only 20% of ruminal microorganisms are able to be cultured; therefore, results obtained *in vitro* may differ greatly from what occurs *in vivo* (Zehavi et al., 2018).

There have also been reports of post-ruminal effects when feeding EFE. Beauchemin et al. (1999) reported feeding EFE to dairy cattle led to increased digestion of starch and greater total tract digestibility of starch, NDF and ADF, which the authors attributed to increased intestinal digestion. Exogenous fibrolytic enzymes may remain active in the small intestine; however, only 2.1% to 5.8% CMCase activity is recovered in the duodenum (Hristov et al., 1998b). Reduced pH of the abomasum combined with pepsin secretion is responsible for this reduction in activity (Hristov et al., 1997). Therefore, it is unlikely activity of EFE within the

distal gastrointestinal tract is responsible for post-ruminal effects. Instead, reduced viscosity of digesta when feeding EFE may allow increased absorption of nutrients in the small intestine, as demonstrated by Hristov et al. (2000) when administering EFE directly into the rumen of cattle. Reductions in duodenal viscosity, however, are not frequently observed when EFE are applied to feed (Hristov et al., 1998b; Yang et al., 2000; Baah et al., 2005). Due to inactivation of EFE and inconsistent effects on intestinal viscosity, post-ruminal impacts of EFE are not considered to be a primary mode of action.

Summary

Application of EFE can be utilized to enhance performance of cattle, including increased amount and quality of milk produced, increased DMI, greater ADG, and reduced FCR. Improved animal performance when feeding EFE is driven by more complete utilization of DM, OM, NDF, and ADF, or any combination of these components. The effect of EFE on digestibility of nutrients and animal performance, however, is impacted by source, rate and method of application of EFE, and dietary composition. Very little is known regarding interactions between these variables. While it is likely pre-ingestive hydrolysis of fiber and synergistic ruminal effects contribute to the physiological responses observed when feeding EFE, variation between experimental methodology confounds available information. A more universal approach regarding application rate, enzyme activity, application method, and basal diet must be adopted in order to elucidate mode of action of EFE. Through consistent experimental methodology, mode of action of EFE may be better understood, providing improved feeding guidelines and possibly producing more consistent effects on nutrient digestibility and animal performance when supplementing EFE.

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Figure 1.1. Carbohydrate binding module (CBM) of cellulolytic ruminal microorganisms (Krause et al., 2003).

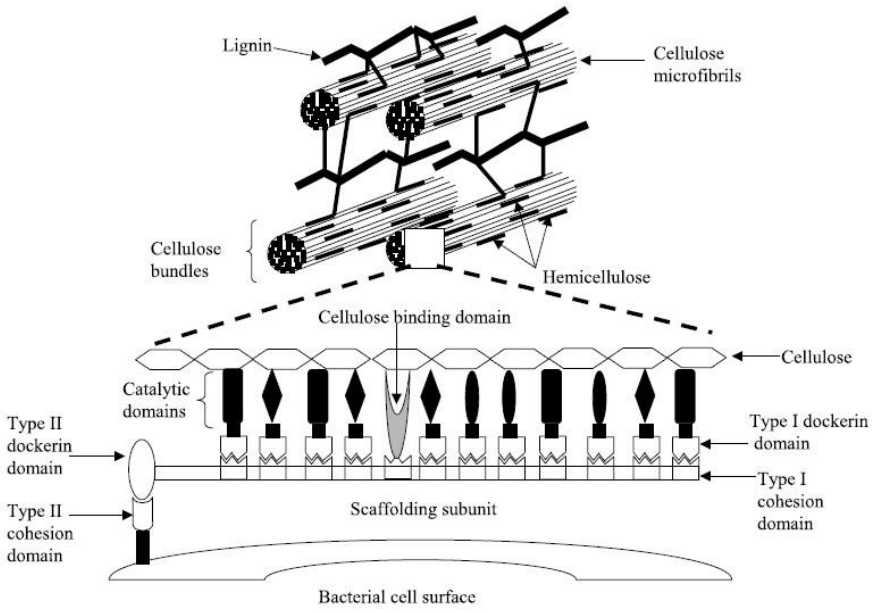


Figure 1.2 Chemical structure of pectin and sites of degradation via enzymes produced by ruminal microorganisms (Müller et al., 2007).

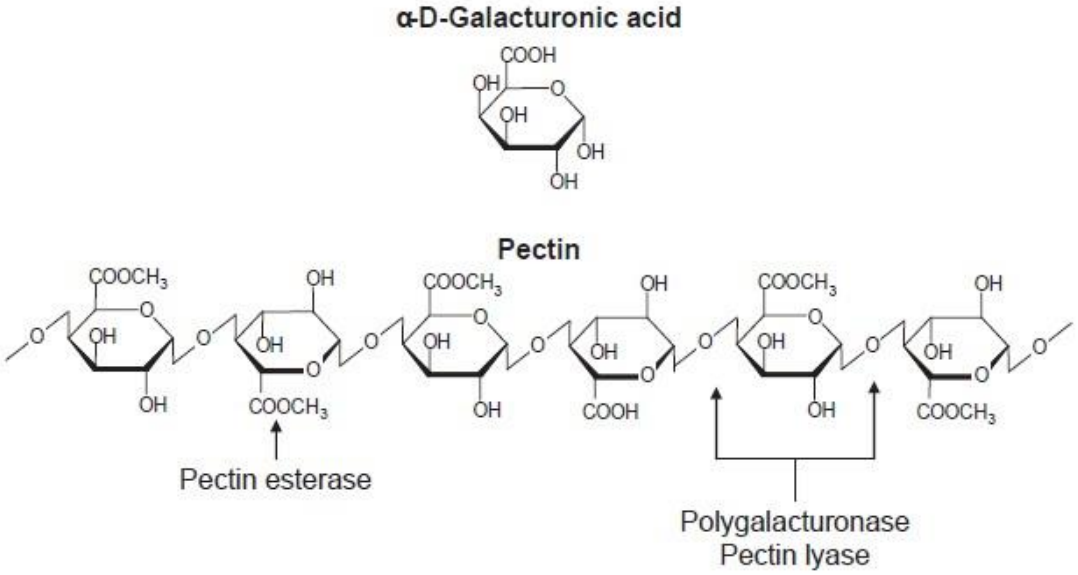
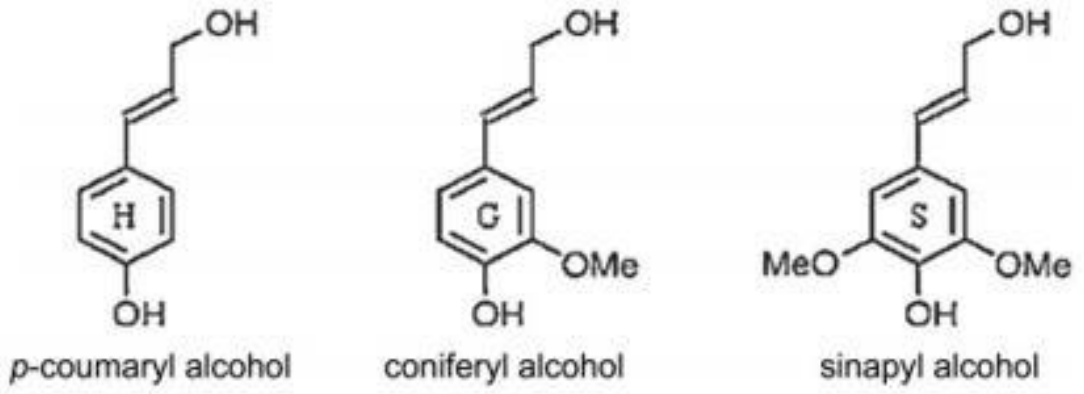
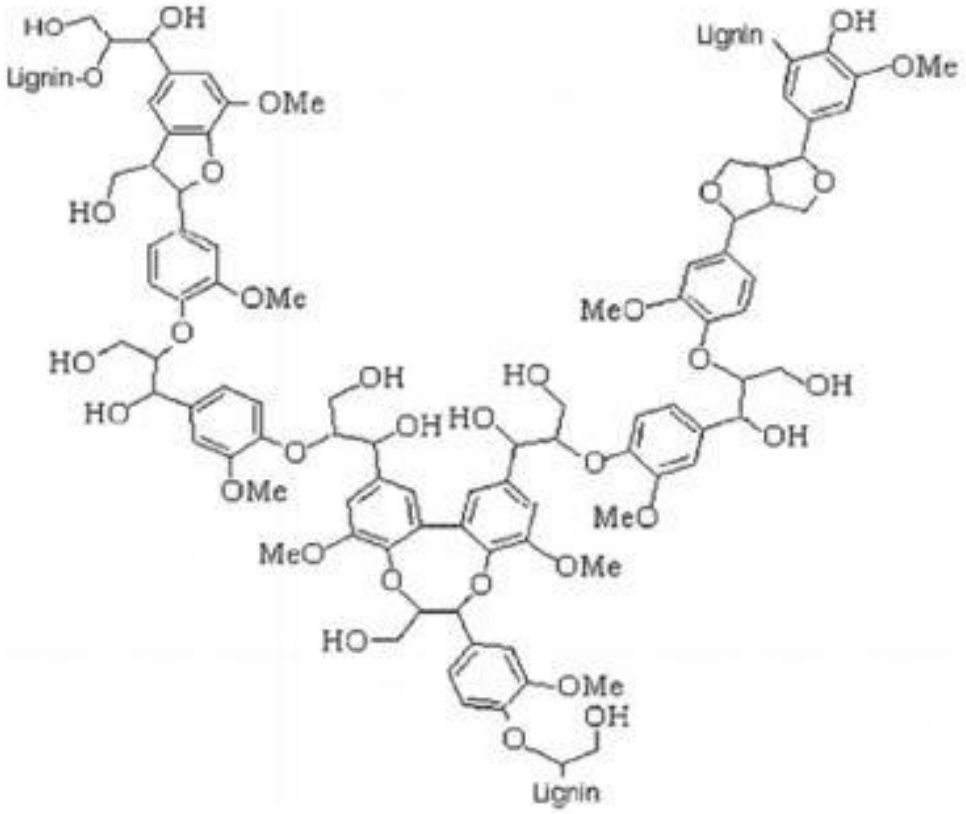


Figure 1.3. Chemical structure of components of lignin[A] and partial chemical structure of lignin[B] (Marković et al., 2012).

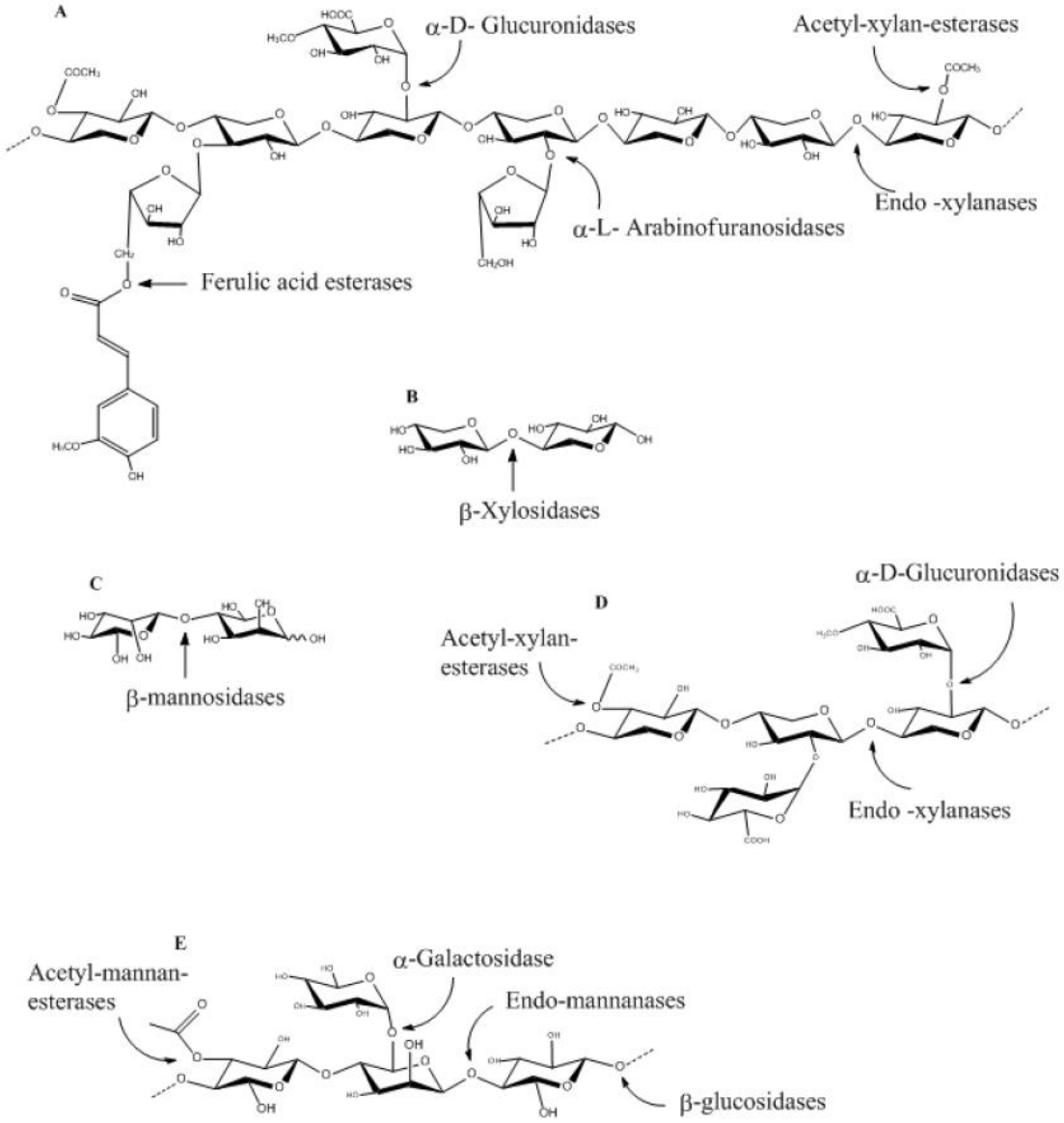


A.



B.

Figure 1.4. Chemical structure of hemicellulose and sites of degradation via enzymes produced by ruminal microorganisms (Conejo-Saucedo et al., 2010).



A. Main structure of hemicellulose and primary ruminal enzymes necessary to cleave side chains.
B. Degradation of xylobiose via endo-xylanases, used to represent degradation of larger xylan chains.
C. D. E. Required enzymes for degradation of side chains of hemicellulose.

Figure 1.5. Chemical structure of cellulose and sites of degradation via enzymes produced by ruminal microorganisms (Yang et al., 2009).

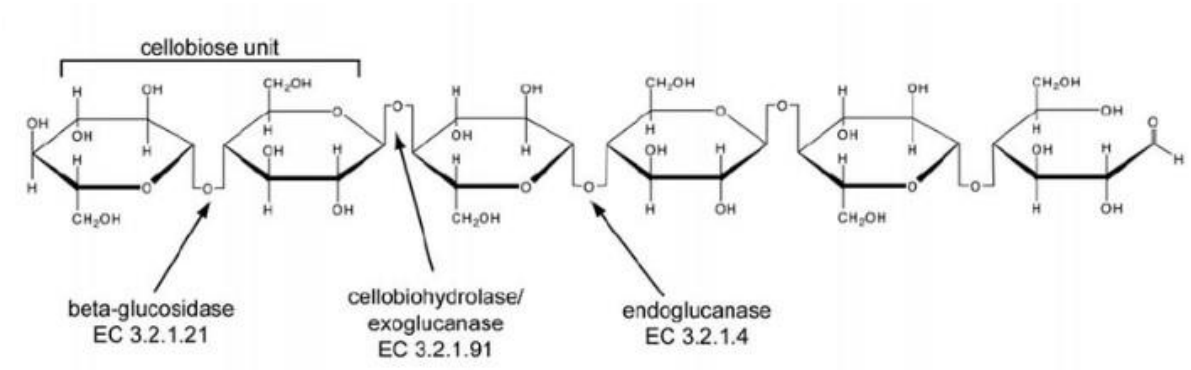


Figure 1.6. Chemical structure of amylose and sites of degradation via enzymes produced by ruminal microorganisms (https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=111642816).

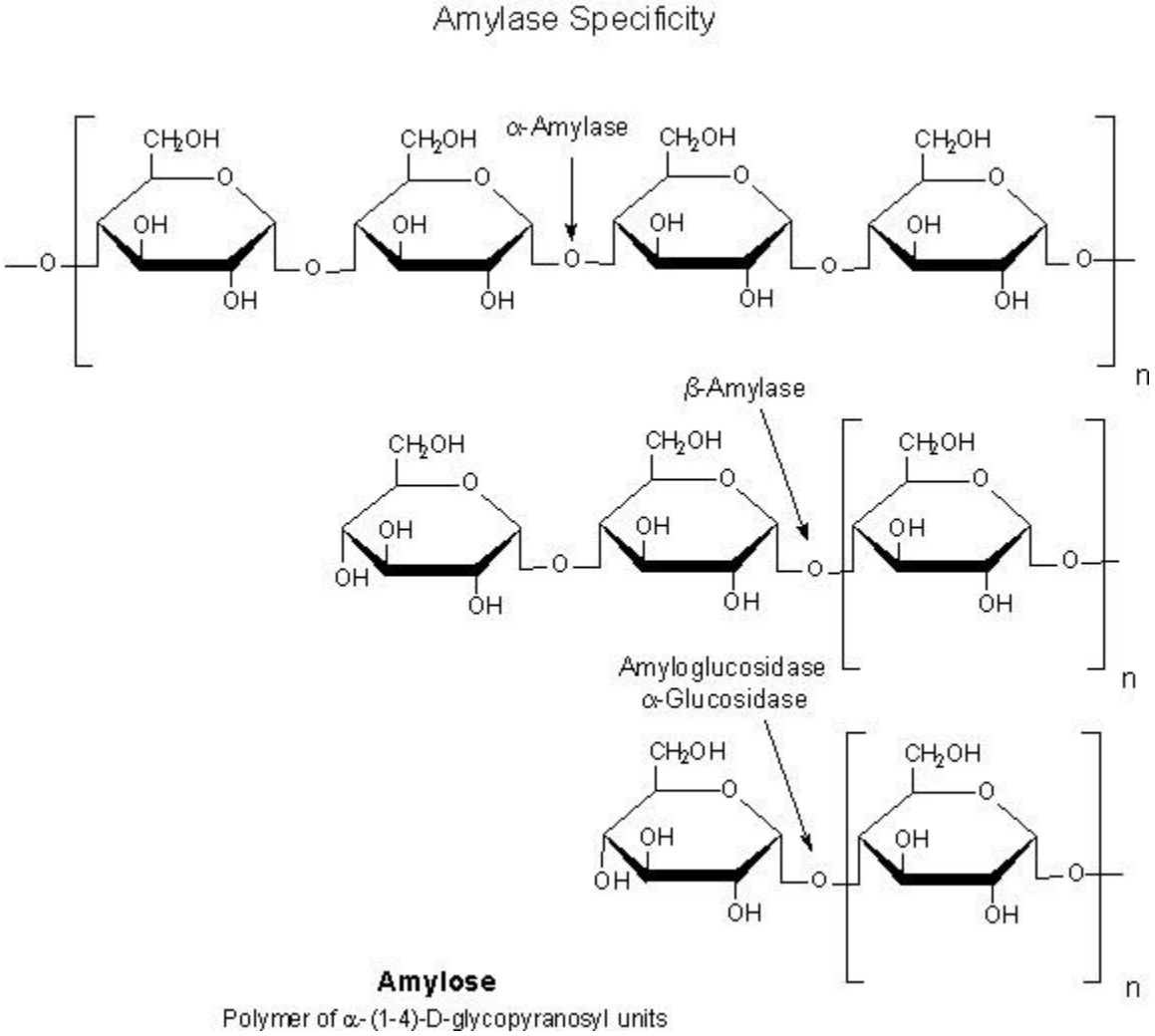
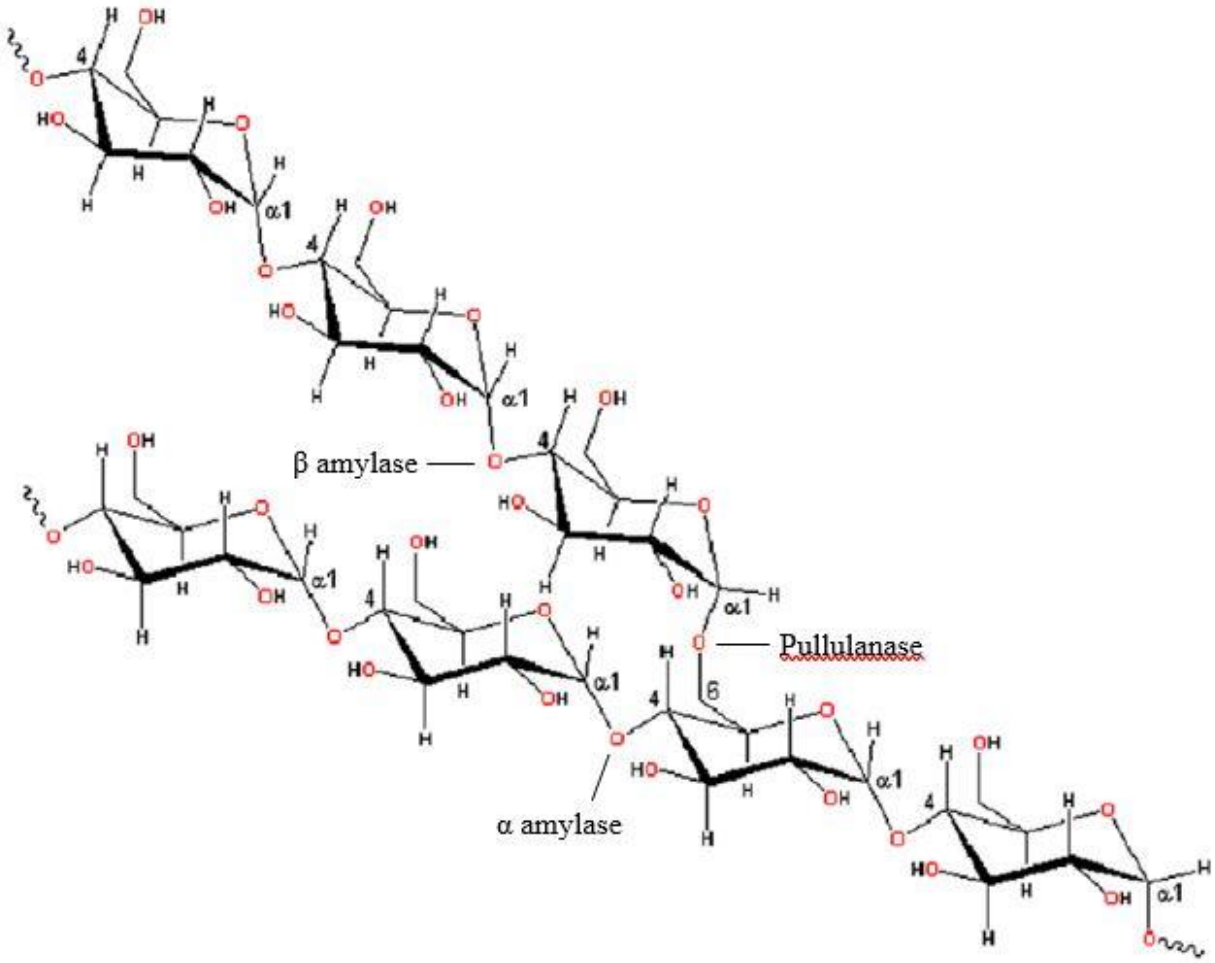


Figure 1.7. Chemical structure of amylose and sites of degradation via enzymes produced by ruminal microorganisms (https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=111642816).



Chapter 2 - Evaluation of different application methods of Vista Pre-T to a dairy TMR on *in vitro* fermentation by bovine ruminal microorganisms

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Abstract

To determine whether application method of exogenous fibrolytic enzymes (EFE) impacts fermentation parameters by bovine ruminal microorganisms, three methods of applying Vista Pre-T (AB Vista, Inc., Plantation, FL.) on a total mixed ration (TMR) were evaluated: sprayed liquid enzyme (wet), liquid enzyme combined with molasses (molasses) prior to mixing molasses into TMR, and dried enzyme added to vitamin and mineral premix (dry) prior to mixing vitamin and mineral premix into TMR. Five grams (dry matter; DM) treated TMR, which were calculated to contain 4.82 mg (DM) Vista Pre-T, were placed into fermentation bottles. Additionally, there also were cultures that received 5 g (DM) untreated TMR with 2.41 mg (DM) liquid Vista Pre-T dosed directly (direct-dosed) into fermentation bottles at the time of inoculation. Negative control cultures contained 5 g (DM) untreated TMR and were not exposed to EFE. One hundred twenty-five milliliters McDougall's buffer and 25 mL ruminal fluid, which served as inoculum, were combined with TMR in fermentation bottles. Bottles were sparged with N_2, fitted with gas pressure monitoring modules, and incubated for 48 h at 39°C. Molasses enzyme application reduced maximum rate of gas production and increased time to reach half maximum gas production compared to all other applications ($P \leq 0.05$). Wet application led to greater terminal pH compared to all other treatments ($P \leq 0.05$). Cultures containing molasses treatment had greater ($P \leq 0.02$) terminal pH compared to dry and direct-dosed treatments. Concentrations of total VFA, acetate, propionate, isobutyrate, and isovalerate were not affected ($P \geq 0.12$) by application method. Acetate:propionate ratio was increased when cultures contained molasses treatment compared to direct-dosed and negative control cultures ($P \leq 0.01$). Butyrate concentrations were greater when cultures contained molasses compared to all treatments ($P \leq 0.009$) except dry ($P = 0.07$). Molasses and dry application methods resulted in

greater valerate concentrations ($P \leq 0.03$) compared to cultures containing wet treatment. Dry application led to greater caproate concentrations compared to all treatments ($P \leq 0.02$) except molasses ($P = 0.40$). *In vitro* dry matter disappearance (IVDMD) was greater for cultures containing dry and molasses treatments than all other application methods ($P \leq 0.0009$). Neutral detergent fiber disappearance (NDFD) was greatest for cultures containing dry application ($P < 0.0001$) with wet application method yielding the lowest NDFD ($P < 0.0001$); however, ranges observed for all parameters were small. While molasses treatment had the greatest impact on dry matter disappearance *in vitro*, this effect has not been confirmed *in vivo*.
Keywords: exogenous fibrolytic enzymes; in vitro; rumen; cattle; TMR

Introduction

Exogenous enzymes have been utilized in the cattle industry since the 1960's to enhance degradation of specific feed components through microbial fermentation (Beauchemin et al., 1997; Beauchemin et al., 2004). These enzymes are typically derived from native ruminal bacteria or fungi cultured in a laboratory setting. The enzyme(s) utilized will vary according to the component targeted for increased fermentation. Commercially available exogenous enzymes generally fall into 1 of 4 categories: proteolytic, lipolytic, saccharolytic, or fibrolytic and degrade proteins, lipids, sugars, or structural carbohydrates (CHO), respectively (Beauchemin et al., 2004). Exogenous fibrolytic enzymes (EFE) such as cellulases and xylanases, which degrade cellulose and hemicellulose respectively, are more commonly utilized than other enzymes due to slow ruminal fermentation of structural CHO. Inclusion of EFE in cattle rations has been reported to improve rate and extent of fermentation of structural CHO which, in turn, increases volatile fatty acid (VFA) production and thus energy available to the animal (Feng et al., 1996; Hristov et al., 2000; Gado et al., 2009; Phakachoed et al., 2013). Others, however, have found no effect on digestibility of fiber when supplementing EFE in dairy and beef cattle rations (Krause et al., 1998; Dhiman et al., 2002; Silva et al., 2016). Theories regarding mode of action for EFE are conflicting. Some suggest application of EFE to forage leads to preingestive hydrolysis, or pitting, resulting in more attachment sites for native ruminal microorganisms, thus promoting a greater rate and extent of fermentation of fiber (Wang et al., 2001; Morgavi et al., 2004). Others suggest activity of EFE is synergistic with native ruminal microorganisms, resulting in greater fermentation responses than expected with either native or exogenous enzymes alone or the additive effect of both (Morgavi et al., 2000; Beauchemin et al., 2003; Gado et al., 2009).

Supplementation with EFE is most beneficial when cattle are in a negative energy state or consuming diets with large quantities of starch (Krause et al., 1998; Zheng et al., 2000; Schingoethe et al., 1999; Beauchemin et al., 2003). Grain-rich diets have been reported to decrease ruminal pH, which generally results in decreased activity of fibrolytic bacteria, thus reducing degradation of structural CHO (Russell and Wilson, 1996; Dijkstra et al., 2012). It is common to feed grain to cattle via total mixed rations (TMR; Schingoethe, 2017). Though formulation will vary with differing animal requirements, most TMR contain approximately 30% to 50% grain. Exogenous enzyme usage varies throughout the industry, from amount of enzyme applied, to component of TMR to which the enzyme is added, to the composition of the animal's diet (Beauchemin et al., 1997; Bowman et al., 2002; Adesogan et al., 2007). This study was designed to evaluate the effect of 3 application methods of a commercially available EFE (Vista Pre-T, AB Vista Inc., Plantation, FL) on *in vitro* fermentation of a standard dairy TMR by mixed ruminal microorganisms.

Materials and methods

Animals

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. Two 3-year-old Holstein-Jersey cross steers previously fitted with ruminal cannulae served as donors of ruminal inoculum. Animals were group housed in a dry lot pen (50 m^2/steer) and offered 17 kg (as-fed) TMR (Table 2.1) head/d. Cattle were maintained on this diet for a minimum of 14 d prior to collection of ruminal fluid.

Experimental design

The study was a randomized complete block design replicated over 2 periods with 2 steers per period and 5 treatments (wet, dry, molasses, direct-dosed, and negative control).

Experimental unit was fermentation bottle. Within period, there were 6 replications (3 per animal) of each treatment. Each period contained 36 fermentation bottles, 6 of which were blanks (ruminal inoculant plus buffer) to account for fermentation of steers' dietary TMR that was collected with ruminal fluid. Values obtained from blanks were subtracted from values obtained from bottles containing treatments and used to generate blank-corrected values for statistical analyses.

Treatment application and sample preparation

A 3,077-kg batch of TMR (Table 2.2) used as substrate during *in vitro* fermentation was prepared using a commercial horizontal auger at the Kansas State University Dairy Teaching and Research Center (Manhattan, KS). Five 25-kg aliquots were collected and treatment (application method) was randomly assigned to each aliquot. Dosing of Vista Pre-T (AB Vista, Inc., Plantation, FL) was calculated using an expected dry matter (DM) of 50%. Each aliquot, except those designated direct-dosed and negative control, received 12.03 g commercial xylanase and cellulase enzyme product (Vista Pre-T; AB Vista, Inc., Plantation, FL; 0.9624 g Vista Pre-T/kg TMR DM). The wet treatment was prepared following the manufacturer's recommendation whereby Vista Pre-T was diluted with water (10:1; water:Vista Pre-T) and sprayed onto the TMR with a commercially available pump sprayer (Chapin International Inc., Batavia, NY) before mixing. For molasses treatment, Vista Pre-T was mixed by hand into 0.25 kg beet molasses and mixed into the TMR. To keep treatments isocaloric, 0.25 kg molasses was added to all aliquots of TMR. The dry treatment included a dried, granular form of Vista Pre-T that was combined with an additional 0.25 kg of the vitamin and mineral premix prior to mixing into the TMR. Accordingly, all other aliquots of TMR also received an additional 0.25 kg of vitamin and mineral premix. A 45.5-kg mixer (H. C. Davis Sons MFG. Co., Inc, Bonner Springs, KS) was

utilized to combine ingredients for 5 min following treatment application and addition of molasses and vitamin mineral premix. Values reported in Table 2.2 include additional vitamin mineral premix and molasses added to TMR aliquots.

Following application of treatments, 1-kg aliquots of each TMR were collected in triplicate according to procedures described by Undersander et al. (2005) for nutrient analyses (Table 2.3) and to serve as substrate for *in vitro* fermentation cultures. Subsamples were frozen at -80°C for a minimum 24 h and ground via a Wiley Mill (4 mm screen; Model 4, Thomas Scientific, Philadelphia, PA). Ground samples were placed in a -80°C freezer for 12 h and ground again with a coffee grinder (Model IDS 55; Mr. Coffee, Boca Raton, FL) to further decrease particle size.

In vitro fermentation

Following grinding, 9.4121 g ± 0.0005 g treated TMR (as-fed; calculated to supply 5 g DM when using an average DM value) were placed into 250-mL fermentation bottles (Ankom Technology Corp., Macedon, NY) containing 125 mL McDougall's buffer (McDougall, 1948) approximately 4 h prior to inoculation with ruminal fluid. Ruminal fluid was collected, strained through 4 layers of cheese cloth, placed into pre-warmed insulated containers, and immediately transported to the laboratory. Ruminal fluid was strained through an additional 8 layers of cheese cloth and placed into N_2-sparged separatory funnels. After 60 min at 39°C in an incubator (Isotemp 550D Incubator Oven; Fisher Scientific, Hampton, NH), ruminal fluid had stratified into 3 layers: a dense sediment layer, an intermediate fluid layer, and a floating mat layer. Twenty-five milliliters of intermediate fluid layer served as inoculum for each bottle. Direct-dosed treatments were prepared by applying 1.94 μL (1.237 mg/μL) liquid Vista Pre-T directly into fermentation flasks at time of inoculation. Thus, bottles containing direct-dosed treatments

received a total of approximately 2.41 mg Vista Pre-T (0.4812 g Vista Pre-T/kg substrate DM), while bottles containing dry, molasses, and wet treatments contained approximately 4.82 mg Vista Pre-T (0.9624 g Vista Pre-T/kg substrate DM). Following inoculation, initial pH was measured using a handheld pH probe (Thermo Orion Star A121; Thermo Fisher Scientific Inc., Chelmsford, MA), bottles were sparged with N_2, and gas pressure monitoring modules (RF Gas Production System; Ankom Technology Corp., Macedon, NY) were fitted on each bottle prior to placement into a 39°C shaking incubator (Model G25; New Brunswick Scientific Inc., New Brunswick, NJ). After 48 h gas pressure monitoring modules were removed, fermentation was terminated by exposure to oxygen, and terminal pH was recorded.

Gas production

Gas pressure was recorded every 15 min in each bottle throughout the 48-h incubation. Data were then converted from pounds per square inch (PSI) into mol of gas produced according to the ideal gas law: $n = p(\frac{V}{RT})$ where n is mol of gas produced, p represents pressure in kPa, V represents L of total headspace in fermentation bottles, R is the gas constant (8.314472 $L \cdot kPa \cdot K^{-1} \cdot mol$), and T represents temperature in Kelvin. Using Avogadro's law, mol of gas produced was multiplied by 22.4 x 1000 (as 22.4 L is the amount of space occupied by 1 mole of gas at 39°C) to determine mL of gas produced (Ankom Technology Corp., 2014). One gas pressure monitoring module (run 1, steer B, wet) malfunctioned and thus data from this bottle were not included in statistical analyses of gas production.

Volatile fatty acids (VFA)

At the conclusion of each 48-h incubation, 4 mL fluid from each bottle were mixed with 1 mL 25% (wt/v) meta-phosphoric acid and frozen at -20°C for a minimum of 24 h. Deproteinized samples were thawed, homogenized (Vortex-Genie 2; Scientific Industries,

Bohemia, NY), and 1.5 mL aliquots were transferred into microcentrifuge tubes and centrifuged (Eppendorf 5415 C; Brinkmann Instruments, Inc., Westbury, NY) for 15 min at 16,000 x g. Approximately 1 mL supernatant was placed into gas chromatography vials with screw caps and placed onto an Agilient 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a Nukol capillary column (#25326 Supelco columns; Sigma-Aldrich, St. Louis, MO) and flame ionization detector (FID). Injection volume of samples was set at 0.2 μL with an injection split ratio of 10:1. Hydrogen served as the carrier gas. Initial oven temperature was 70°C and increased by 30°C/min until a final temperature of 190°C was reached and held for 2 min. Inlet and FID temperature were set at 300°C. Internal pressure was maintained at 1.0749 PSI and flow rate was 5.2814 mL/min. Total run time was 6 min per sample. Comparison to a VFA standard solution (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO) was utilized to quantify individual VFA present in samples.

In vitro dry matter disappearance (IVDMD) and neutral detergent fiber disappearance (NDFD)

Remaining contents from each bottle were poured into aluminum pans and placed in a 55°C forced air oven for 4 d to determine IVDMD according to the following equation: $\frac{\mathrm{Ws} - [(\mathrm{Wds} - \mathrm{Wp}) - (\mathrm{Wc} - \mathrm{Wp})]}{\mathrm{Ws}} \times 100$. Ws represents g (DM) TMR originally weighed into fermentation bottles, Wds is weight of dried sample and pan, and Wp represents empty weight of individual pans prior to addition of culture contents. Wc is weight of the contents from the blank and pan assigned to the same replicate as the sample for which IVDMD is being calculated and was used to correct for the weight contribution of buffer and ruminal fluid.

Residual matter was removed from pans and ground by hand using a mortar and pestle. Samples were then placed into plastic bags until neutral detergent fiber (NDF) analyses. One half

gram of each ground sample was placed into Ankom fiber filter bags in duplicate to analyze NDF according to methods described by Goering and Van Soest (1970) without sodium sulfite or heat stable α-amylase (Van Soest et al., 1991; Mertens, 2002; Udén et al., 2005; Gomes et al., 2012) in an Ankom^{200} fiber analyzer (Ankom Technology Corp., Macedona, NY). To determine total g NDF initially available in each bottle, nonfermented TMR for each treatment was also subjected to NDF analysis. Each run contained 1 empty fiber bag to determine baseline changes in weight. Percent NDFD was calculated by using the equation Ws - $\frac{Wf-(Wds-Wb)}{Wf} \times 100$ (Goering and Van Soest, 1970) where Ws is g NDF present in substrate prior to fermentation, Wf represents g substrate weighed into fiber bags prior to NDF analysis, Wds represents residual g DM in the sample bag following NDF analysis, and Wb represents bag weight.

Statistical analyses

Production of VFA, terminal pH, IVDMD, and NDFD data were analyzed using the MIXED procedure of SAS (Version 9.4). Models for *in vitro* VFA production, terminal pH, IVDMD, and NDFD included fixed effect of treatment (application method) and random effect of replicate nested within run. Experimental unit was fermentation bottle. Gas production data were analyzed using Proc GLIMMIX (SAS version 9.4) and included fixed effects of application method and random effects of steer, replicate, and run. Gas production data were modeled using the log logistic distribution in SÃS NLIN. Log logistic modeling was performed using the equation: $\left(\frac{\boldsymbol{K}}{\boldsymbol{1+exp\ (-\beta\ (t-t_{half})}}\right) + \boldsymbol{\varepsilon}$, where K represents maximum production of gas, t_{half} is the time required to reach half of K, β is the log logistic growth rate, and ε is the error term. Parameters generated form NLIN modeling were then analyzed using Proc MIXED of SÃS (version 9.4) to evaluate effects of application method on K, t_{half}, and β. Pairwise comparisons

were evaluated via the 2-sided test for non-zero differences at the 0.05 significance level and tendencies were declared at $0.05 < P < 0.10$.

Results

Gas production

There were main effects of application method ($P < 0.0001$; Table 2.4; Fig. 2.1) on time to achieve half the maximum gas production and the maximum rate of gas production. Maximum gas production, however, was not affected by application method ($P = 0.3506$). Cultures containing wet application achieved half maximum gas production faster than all other treatments ($P \leq 0.05$), except dry application ($P > 0.05$). Cultures containing molasses application method required the longest time to achieve half maximum gas production ($P \leq 0.05$). Maximum rate of gas production was greatest in cultures containing wet application treatments and was most reduced in cultures containing molasses application treatments ($P \leq 0.05$).

Terminal pH

There was an effect of application method ($P = 0.0001$; Table 2.5) on terminal pH. Cultures containing wet treatment had greater pH compared to those with negative control ($P = 0.0009$), direct-dosed ($P < 0.0001$), molasses ($P = 0.0494$), and dry ($P < 0.0001$) treatments. Terminal pH was greater in cultures containing molasses application than dry ($P \leq 0.0127$) and direct-dosed ($P \leq 0.0204$) cultures.

VFA

There was not a main effect ($P \geq 0.1208$) of application method on total VFA, acetate, propionate, isobutyrate, or isovalerate. There was a tendency for heptanoate to be affected by application method ($P = 0.0512$). Butyrate, valerate, caproate, and acetate:propionate ratio (A:P)

were affected ($P \leq 0.0443$) by application method. Cultures containing molasses treatment had increased A:P compared to direct-dosed ($P = 0.0094$) and negative control ($P = 0.0117$). Wet application tended to lead to increased A:P compared to direct-dosed ($P = 0.0774$) and negative control ($P = 0.0920$) cultures. Molasses treatment led to increased butyrate concentrations compared to negative control ($P = 0.0091$), direct-dosed ($P = 0.0065$), and wet application ($P < 0.0001$) and butyrate tended to be greater than in cultures with dry treatments ($P = 0.0744$). Meanwhile, cultures containing wet treatment had reduced butyrate concentrations compared to all other treatments ($P \leq 0.0240$). Valerate concentrations were greater in cultures with molasses ($P = 0.0024$) and dry ($P = 0.0255$) treatments compared to those containing wet application. There was a tendency for valerate concentrations to be reduced in cultures with wet application compared to negative control ($P = 0.0872$). Dry application led to greater concentrations of caproate compared to negative control ($P = 0.0142$), direct-dosed ($P = 0.0047$), and wet ($P = 0.0002$) treatment. Caproate concentrations were greater in molasses treated cultures compared to wet ($P = 0.0029$) and direct-dosed ($P = 0.0390$) cultures and tended to be greater ($P = 0.0957$) compared to negative control.

IVDMD and NDFD

Both IVDMD and NDFD were affected by application method ($P < 0.0001$) and IVDMD was also affected by replicate ($P = 0.0106$). Cultures containing dry and molasses treatments had increased ($P \leq 0.0009$) IVDMD compared to all other cultures. Inclusion of dry enzyme yielded the greatest ($P < 0.0001$) NDFD while the wet application had the least ($P < 0.0001$). Cultures containing molasses treatment did not differ ($P \geq 0.5757$) from negative control nor direct-dosed cultures for NDFD.

Discussion

When considering data collected regarding gas production, IVDMD, and NDFD, it appears that dry treatments most enhanced microbial activity and were most effective at improving fermentation of fiber. Gas production, however, does not always correlate with extent of fermentation, as differences in microbial communities may alter rate and composition of gas production without necessarily influencing total substrate disappearance (Beuvink, 1993; Lopez et al., 1999). This may be what occurred in molasses-treated cultures whereby maximum rate of gas production was reduced and time to achieve half maximum gas production was increased compared to all other treatments, but IVDMD was not different in cultures containing molasses application compared to dry application. Despite similarities in IVDMD, cultures containing dry treatment had approximately 4% greater NDFD compared to cultures with molasses treatment, indicating increased fermentation of structural carbohydrates with dry application. This is an important distinction as EFE are intended to increase rate of fiber degradation in the rumen. The addition of granular EFE through vitamin and mineral premixes or concentrates mixed into TMR in a manner similar to dry treatment in the current experiment has been demonstrated *in vitro* and *in vivo* to increase disappearances of organic matter, dry matter, NDF, and acid detergent fiber (ADF) when compared to negative controls (Bowman et al., 2002; Rode et al., 1999). While addition of EFE to TMR through molasses is less common, we were not the first to use this technique. Clyburn (1999) reported no effect of a molasses-based EFE treatment on nutrient digestibility when fed to steers, but rate of passage through the rumen may have limited the efficacy of EFE. In the present study, cultures containing molasses did not appear to increase rate of fermentation. The reason for this slower rate of fermentation is unknown. This decreased rate

of fermentation, however, may only be relevant with shorter fermentation periods, as total IVDMD was not reduced after 48 h of fermentation.

There was reduced ADF and NDF in TMR treated with EFE via molasses when compared to TMR subjected to other treatments (Table 2.3). This may be due to greater hydrolysis of fiber by the EFE, which should increase initial availability of simple sugars and potentially shift the enzymes released from ruminal microorganisms. Many fiber degrading microorganisms can degrade short chains of cellulose and hemicellulose and will preferentially ferment these shorter chains compared to larger and more complex molecules (Fusee and Leatherwood, 1972). Subsequently, there is a reduction in ruminal concentrations of xylanases and cellulases. In addition, if more sugars were available earlier in the incubation, saccharolytic bacteria would be expected to proliferate in these cultures and possibly out-compete fibrolytic bacteria for nutrients. However, rate of gas production during the fermentation in cultures with molasses application was slower compared to dry application, which is in direct conflict with expectations of fermentation when greater quantities of rapidly fermentable sugars are present. Lastly, preingestive hydrolyzation of cross-linkages between lignin and hemicellulose of forage may have liberated phenolic compounds which are toxic to some ruminal microorganisms and thus may reduce degradation of cellulose and hemicellulose (Chesson et al., 1982; Jung, 1985). This could account for reduced NDFD in cultures containing molasses treatment compared to cultures containing dry treatment, while IVDMD did not differ.

A reduction in NDFD in cultures containing wet application was unexpected, as EFE generally increases or has no impact on fiber degradation. With wet application, EFE was initially applied to a greater proportion of feed than other treatments as it was not restricted to molasses or concentrate. While it was initially theorized this should lead to greater hydrolysis of

fiber prior to fermentation than other treatments, this is not supported by the analyses of TMR used as substrate for cultures (Table 2.3). Interestingly, cultures containing wet application achieved half maximum gas production faster than all treatments except dry and had the greatest maximum rate of gas production, which is direct contrast to *in vitro* dry matter and neutral detergent fiber disappearance. Wang et al. (2001) reported diluted EFE sprayed onto TMR led to increased *in vitro* degradation of rolled barley grain; however, degradation of chopped alfalfa was not affected. Conversely, Kung et al. (2000) found spraying 5 L of EFE diluted with 5 L of water onto 1,000 kg forage led to increased NDFD when feeding TMR to dairy cows. This illustrates the need for further research concerning rate of application, source of EFE, type of feed to which EFE is applied, and effect of *in vivo* vs. *in vitro* research techniques on results obtained.

With moderate rates of gas production in cultures containing dry treatments and increased IVDMD in cultures containing dry and molasses treatments compared to all other application methods, it was expected total VFA would be increased in these cultures. This, however, was not observed, possibly due to small differences in substrate fermentation measured via IVDMD across all treatments (49.05% to 50.70%). There were minor differences between individual VFA, indicating treatment application influenced portion of feed degraded or led to shifts in microbial populations, as individual VFA production varies depending on substrate and which microorganisms are present (Stewart et al., 1988). Butyrate was the VFA most impacted and is of particular importance as it is metabolized within the rumen to maintain a healthy lumen (Vital et al., 2014). Butyrate is a primary VFA produced during starch degradation (Vital et al., 2014). Cultures containing molasses treatments produced the greatest butyrate concentrations, and therefore, likely had greater fermentation of soluble CHO. This is supported by cultures with

molasses application having the greatest IVDMD but similar NDFD as negative control, indicating greater fermentation of soluble components of substrate. Cultures containing dry treatments had the greatest fiber degradation, which is expected to increase acetate. This was not observed in the present study; however, acetate is interconverted to butyrate and propionate in the rumen (Bergman et al., 1965), which may have occurred in the present study. It is also important to note VFA concentrations obtained in this study offer only a glimpse at terminal conditions in cultures and do not fully account for production nor utilization of VFA by microorganisms or absorption by the animal.

It has been well established ruminal VFA concentration is the driving factor behind pH (Dijkstra et al., 2012). Accordingly, a lack of differences in concentrations of total VFA corresponded with a narrow range in pH, ranging from 5.35 to 5.39. These differences in pH would have very limited, if any, impact on microbial fermentation. While fermentation of fiber is greatest at pH 6 to 7 (Erfle et al., 1982; Hoover, 1986), there are no differences in numbers of cellulolytic bacteria when pH is reduced from 5.5 to 5 (Erfle et al., 1982). A similar effect occurs with cellulase activity, as it is slightly decreased when pH is reduced from 5.5 to 5, but there is drastic reduction of activity with pH < 5 (Kitts and Underkofler, 1954).

Our observations that direct-dosed treatments did not alter any fermentation parameters compared to negative control cultures may have been due to insufficient concentration of Vista Pre-T in direct-dosed cultures. Hristov et al. (2000), however, reported no difference in ruminal fermentation of NDF and decreased efficiency of DM fermentation when dosing EFE directly into the rumens of cannulated heifers. Thus, it appears EFE are most effective when administered via feed, supporting a preingestive mode of action as opposed to merely providing ruminal microorganisms with growth factors. Proximate analyses of TMR in the current study revealed

dry and molasses application methods may have led to degradation of NDF and ADF in TMR before fermentation was initiated. This was unexpected, as samples were immediately transported to the laboratory and frozen following treatment application. Freezing samples was necessary for grinding of TMR and proper storage until the *in vitro* fermentation period, however it likely limited preingestive activity of enzymes on TMR. While it is possible repeated freeze-thaw cycles necessary for sample processing altered enzyme activity (Hashimoto et al., 1971; Sinsabaugh and Linkins, 1989), all treatments were subjected to the same processing protocol and, therefore, any resulting changes in EFE activity were expected to be similar across treatments.

Of course, there are important differences to consider between *in vitro* and *in vivo* experiments. Use of batch culture fermentation in the present study may have disguised some of the effects associated with application of exogenous enzymes, as finite substrate is provided. Greater impact may be observed *in vivo* where substrate is renewed and end products of fermentation are absorbed and utilized by the animal. Because only approximately 20% of bacteria within the rumen are culturable, results may differ in the animal where additional microorganisms are present (Zehavi et al., 2018).

Conclusion

Dry and molasses application of Vista Pre-T appeared to be the most beneficial in increasing disappearance of a dairy TMR by cultured bovine ruminal microorganisms. If supplementing EFE to increase fiber utilization in cattle with rapid rates of passage, however, the dry application method may be superior based on increased rate of gas production in the present study based on a more rapid rate of fermentation compared to EFE applied to molasses. While

this experiment provides valuable preliminary information, more research evaluating application method of EFE on *in vivo* fermentation is warranted.

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Table 2.1. Composition of total mixed ration (TMR) fed to steers[1,2]

Ingredient	Amount, %
Corn silage	46.67
Sweet Bran[3]	19.67
Dry rolled corn	16.67
Prairie hay	14.67
Supplement[4]	2.00

[1]Steers serving as donors of ruminal fluid were offered 17 kg (as-fed) TMR animal/d.
[2]Mixed on site at the Dairy Teaching and Research Center, Kansas State University, Manhattan, KS.
[3]Cargill Incorporated, Minneapolis, MN.
[4]Supplement contained approximately 46.7% limestone, 34.6% urea, 9.7% potassium chloride, 7.1% sodium chloride, 1.1% vitamin and mineral mix, 0.2% vitamin A, 0.1% vitamin E, and 0.5% Rumensin (Elanco Animal Health, Greenfield, IN) and was manufactured at the O. H. Kruse Feed Technology Innovation Center, Kansas State University, Manhattan, KS.

Table 2.2. Composition of total mixed ration (TMR) used as substrate for in vitro fermentation[1]

Ingredient	Inclusion, %[*]
Vitamin and mineral premix[2]	28.83
Corn silage	24.80
Sweet Bran[3]	21.36
Alfalfa hay	13.20
Cottonseed hulls	3.51
Triticale silage	3.22
Cracked corn	2.20
Prairie hay	1.91
Molasses	0.98

[1]0.063 g/mL (as-fed) provided to mixed bovine ruminal microorganisms cultured for 48 h.

[*]Includes additional 0.25 kg vitamin and mineral premix and 0.25 kg molasses added to all TMR aliquots. This was necessary as 0.9624 g granulated Vista Pre-T (AB Vista, Inc., Plantation, FL.)/kg dry matter TMR was mixed into additional vitamin and mineral premix or molasses before incorporation into TMR for the dry and molasses treatments, respectively.

[2]Contained ground corn, Megalac R (Church & Dwight, Princeton, NJ), trace mineral salt, magnesium oxide, Lactation PMX containing sodium bicarbonate, potassium chloride, calcium salts of long chain fatty acids, vitamin A supplement, vitamin D3 supplement, biotin, ethylenediamine dihydroiodide, sodium selenite, mineral oil, and calcium carbonate (O. H. Kruse Feed Technology Innovation Center, Kansas State University, Manhattan, KS), Zinpro 4 Plex and Zinpro 120 (Zinpro, Eden Prairie, MN), vitamin E, salt, Soy Plus (Landus Cooperative, Ames, IA) and Rumensin (Elanco Animal Health, Greenfield, IN). Manufactured at the O. H. Kruse Feed Technology Innovation Center, Kansas State University, Manhattan, KS.

[3]Cargill Incorporated, Minneapolis, MN.

Table 2.3. Proximate analysis[1] (dry matter basis) of total mixed rations (TMR) provided as substrate to bovine ruminal cultures

Item	Negative control[3]	Vista Pre-T treatment[2]			
		Direct-dosed[4]	Dry[5]	Wet[6]	Molasses[7]
Dry matter, %	51.1	51.1	51.2	50.5	50.9
Neutral detergent fiber, %	32.5	32.5	31.3	34.0	29.0
Acid detergent fiber, %	19.5	19.5	18.1	19.3	17.0
Crude protein, %	18.0	18.0	18.3	18.3	18.2
NEm, mcal/kg[8]	1.70	1.70	1.71	1.68	1.74

[1]Proximate analysis using wet chemistry (Dairy One Forage Lab, Ithaca, NY).
[2]AB Vista Pre-T (AB Vista, Inc., Plantation, FL), an exogenous fibrolytic enzyme.
[3]No exposure to Vista Pre-T.
[4]0.4812 g Vista Pre-T/kg dry matter TMR applied directly into fermentation bottles.
[5]0.9624 g granulated Vista Pre-T/kg dry matter TMR added to vitamin and mineral premix before incorporation into TMR.
[6]0.9624 g liquid Vista Pre-T/kg dry matter TMR was diluted with water (10:1; water: Vista Pre-T), sprayed directly onto the TMR, and mixed.
[7]0.9624 g liquid Vista Pre-T/kg dry matter TMR was mixed with 0.25 kg liquid molasses before being mixed into TMR.
[8]Net metabolizable energy for maintenance.

Table 2.4. Effect of Vista Pre-T[1] application method on maximum gas production (*K*), time to reach half maximum gas production (t_{half}), and maximum rate of gas production (β) in cultures of bovine ruminal microorganisms provided with treated total mixed rations (TMR)[‡]

Item	Negative control[2]	Vista Pre-T treatment				SEM	*P* -value
		Direct-dosed[3]	Dry[4]	Wet[5]	Molasses[6]		
K	596.21	609.38	613.99	589.04	608.04	28.5473	0.3506
t_{half}	10.3003[b]	10.2505[b]	9.9843[ab]	9.7327[a]	11.0510[c]	0.4041	< 0.0001
β	0.2492[b]	0.2444[b]	0.2468[b]	0.2644[c]	0.2216[a]	0.006	< 0.0001

[1]AB Vista Pre-T (AB Vista, Inc., Plantation, FL), an exogenous fibrolytic enzyme.

[‡]Cultures contained 0.063 g/mL (as-fed) of a dairy TMR and 25 mL strained bovine ruminal fluid as microbial inoculum in 48-h batch culture fermentation.

[2]No exposure to Vista Pre-T.

[3]0.4812 g Vista Pre-T/kg dry matter TMR applied directly into fermentation bottles.

[4]0.9624 g granulated Vista Pre-T/kg dry matter TMR added to vitamin and mineral premix before incorporation into TMR.

[5]0.9624 g liquid Vista Pre-T/kg dry matter TMR was diluted with water (10:1; water: Vista Pre-T), sprayed directly onto the TMR and mixed.

[6]0.9624 g liquid Vista Pre-T/kg dry matter TMR was mixed with 0.25 kg liquid molasses before being mixed into TMR.

[7]Acetate:propionate ratio.

[a,b,c]Means within a row with a different superscript differ ($P < 0.05$).

Table 2.5. Effect of Vista Pre-T[1] application method on terminal pH, volatile fatty acid concentrations (VFA), in vitro dry matter disappearance (IVDMD), and neutral detergent fiber disappearance (NDFD) in cultures of bovine ruminal microorganisms provided with treated total mixed rations (TMR)[‡]

Item	Vista Pre-T treatment						
	Negative control[2]	Direct-dosed[3]	Dry[4]	Wet[5]	Molasses[6]	SEM	*P*-value[ɬ]
pH	5.36[ab]	5.35[a]	5.35[a]	5.39[c]	5.38[b]	0.0087	A
Acetate, mm	76.643	76.155	78.113	78.554	79.226	2.6846	NS
Propionate, mm	55.964	55.762	56.396	56.328	56.110	1.6936	NS
A:P[7]	1.366[a]	1.365[a]	1.383[ab]	1.396[ab]	1.412[b]	0.0172	A
Isobutyrate, mm	0.653	0.641	0.648	0.659	0.691	0.0256	NS
Butyrate, mm	12.695[b]	12.643[b]	13.068[bc]	11.696[a]	13.800[c]	0.4050	A
Isovalerate, mm	0.824	0.802	0.821	0.819	0.891	0.0348	NS
Valerate, mm	3.218[ab]	3.208[ab]	3.275[b]	3.040[a]	3.368[b]	0.1018	A
Caproate, mm	0.367[ab]	0.362[a]	0.398[c]	0.349[a]	0.388[bc]	0.0122	A
Heptanoate, mm	0.013	0.009	0.022	0.013	0.018	0.0044	NS
Total VFA, mm	150.38	149.58	152.74	151.46	154.49	4.8028	NS
IVDMD, %	49.05[a]	49.27[a]	50.67[b]	49.12[a]	50.70[b]	0.3938	A, R
NDFD, %	60.56[b]	60.36[b]	64.42[c]	56.77[a]	60.26[b]	0.5370	A

[1]AB Vista Pre-T (AB Vista, Inc., Plantation, FL), an exogenous fibrolytic enzyme.

[‡]Cultures contained 0.063 g/mL (as-fed) of a dairy TMR and 25 mL strained bovine ruminal fluid as microbial inoculum in 48-h batch culture fermentation.

[2]No exposure to Vista Pre-T.

[3]0.4812 g Vista Pre-T/kg dry matter TMR applied directly into fermentation bottles.

[4]0.9624 g granulated Vista Pre-T/kg dry matter TMR added to vitamin and mineral premix before incorporation into TMR.

[5]0.9624 g liquid Vista Pre-T/kg dry matter TMR was diluted with water (10:1; water: Vista Pre-T), sprayed directly onto the TMR, and mixed.

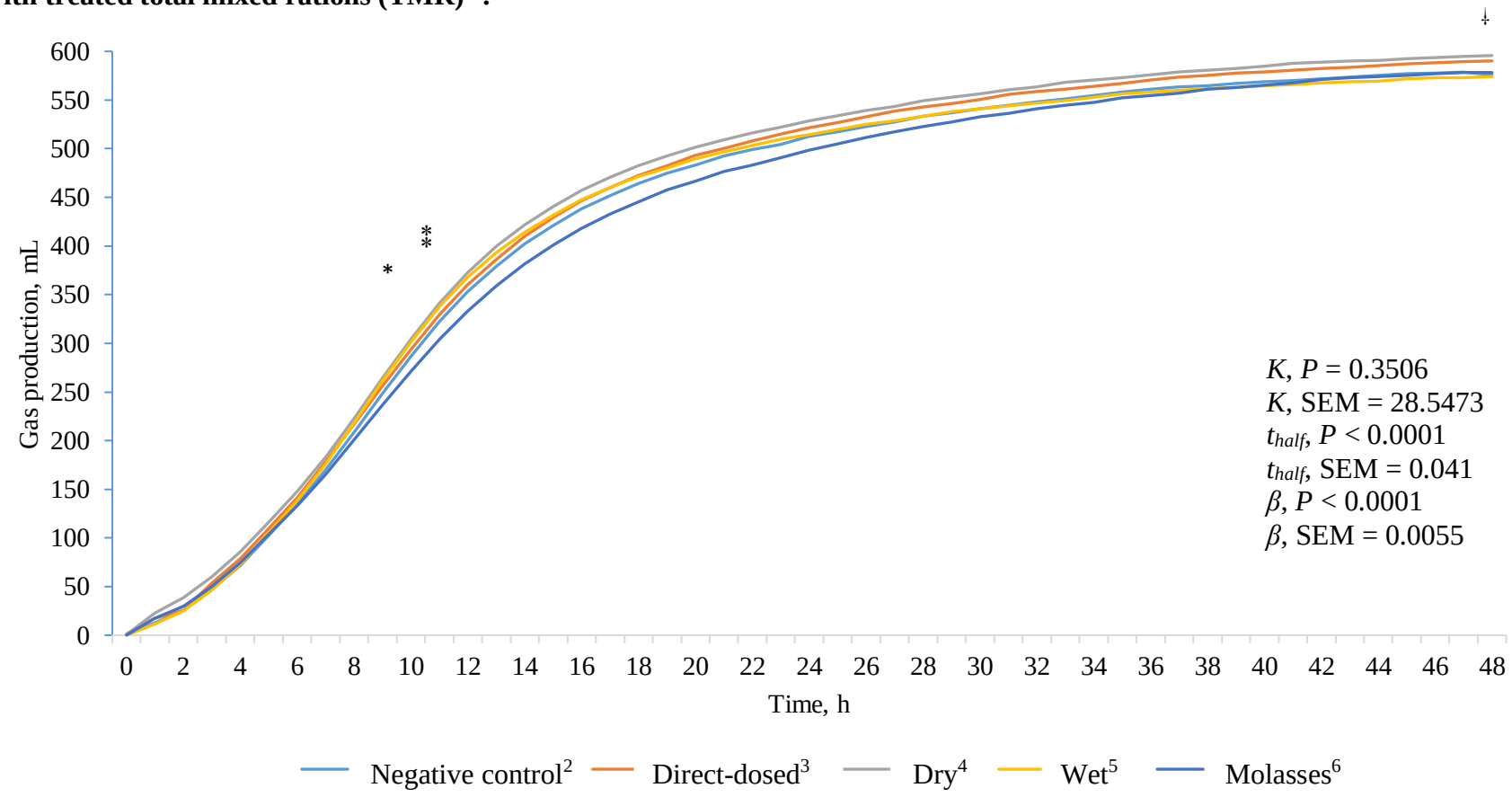
[6]0.9624 g liquid Vista Pre-T/kg dry matter TMR was mixed with 0.25 kg liquid molasses before being mixed into TMR.

[7]Acetate:propionate ratio.

[ɬ]A = effect of application ($P < 0.05$); R = effect of replicate ($P < 0.05$); NS = no main effects ($P > 0.05$).

[a,b,c]Means within a row with a different superscript differ ($P < 0.05$).

Figure 2.1. Effect of Vista Pre-T[1] application method on in vitro gas production by bovine ruminal microorganisms provided with treated total mixed rations (TMR)⁂.



⁂Cultures contained 0.063 g/mL (as-fed) of a dairy TMR and 25 mL strained bovine ruminal fluid.
[1]AB Vista Pre-T (AB Vista, Inc., Plantation, FL), an exogenous fibrolytic enzyme.
[2]No exposure to Vista Pre-T.
[3]0.4812 g liquid Vista Pre-T/kg dry matter TMR applied directly into fermentation bottles.
[4]0.9624 g granulated Vista Pre-T/kg dry matter TMR was added to a vitamin and mineral premix before being added to the TMR.
[5]0.9624 g liquid Vista Pre-T/kg dry matter TMR was diluted with water (10:1), sprayed directly onto the TMR, and mixed.
[6]0.9624 g liquid Vista Pre-T/kg dry matter TMR was mixed into 0.25 kg liquid molasses before being applied to the TMR.

[*]Cultures containing wet application achieved time to half maximum gas production (t_{half}) more quickly than all other treatments ($P \leq 0.05$) expect dry application

[‡]Cultures containing molasses application achieved time to half maximum gas production (t_{half}) more slowly than all other treatments ($P \leq 0.05$).

[ǂ]Application method had no effect on maximum gas production (K; $P = 0.3506$).

Chapter 3 - Literature review: Pelleting, palatability, and flavoring agents in the equine ration

Introduction

Fruit products have been incorporated into companion animal diets for several years, serving as sources of natural antioxidants (Dunlap et al., 2006). Blueberries have been included in pet foods largely due to owner perception of their superfruit properties. This market, however, remains unexplored in the equine industry and there are no commercially available horse feeds that contain blueberry products. A primary obstacle to inclusion of liquid ingredients, such as blueberry products, in equine rations is preserving pellet quality and durability while maintaining an acceptable pellet:fine ratio. Too many fines lead to a variety of issues, including increased production costs, feed wastage, nutrient deficiencies, and respiratory issues (Henry and Morrison, 1923; Ivester et al., 2014).

Another potential obstacle to including blueberry products in equine rations is neophobic response and subsequent feed refusal, which is often observed when new feed is offered (Lindberg and Karlsson, 2001; van den Berg et al., 2016a). Pairing new ingredients with familiar flavors or odors may attenuate neophobic response (van den Berg et al., 2016a). Horses accept a variety of flavoring agents, with preference for sweet flavors (Randall et al., 1978; Goodwin et al., 2005a); however, acceptability of blueberries by horses remains untested.

This review will address factors affecting pellet durability with particular focus on the impact of liquid additives. Equine acceptance and preference of various flavoring agents also will be explored.

History of pelleted feeds

The importance of animal feed has been understood for centuries, with development of intentional feeding programs before people began writing (Coffey et al., 2015). Early feeding systems were largely based on tradition and availability of ingredients, rather than nutritional requirements (Henry, 1913). It was not until the 1800's that improved understanding of nutritional requirements led to advancement in feeding systems (Henry, 1913). Wolff published the first feeding standard based on digestible nutrients in 1864 (Bursiek, 2005), which led to the use of mixed grains in livestock rations. McCampbell (1917) was one of the earliest researchers to evaluate the effect of feeding multiple grains on equine performance. He reported horses consuming a mix of corn, bran, and linseed oil meal had similar condition and spirit as horses consuming oats, the feeding standard of the time, while the mixed feed cost less.

Mixed rations were often fed as mixed whole grains or mashes. It was not until the 1920's that pelleted feed became commercially available (Coffey et al., 2015). Pelleted feeds became more complex throughout the 1900's as micro ingredients such as vitamins and minerals were added. Many changes in feed formulation were driven by the publication of the 1944 National Research Council's (NRC) feeding standards for livestock and poultry. By 1975, feed mill operations were becoming automated, and these systems served as the basis for current feed manufacturing systems (Bursiek, 2005; Coffey et al., 2015). More recent advancements have improved pellet durability while reducing manufacturing costs.

Benefits of processing equine feeds

Increased digestibility and weight gain were some of the earliest benefits associated with feeding ground grain rather than whole grains to horses (Morrison et al., 1919; Caine, 1936; Kienzle et al., 1997). Grinding increases surface area, enhancing enzymatic digestion of starches,

thus allowing for more complete pre-cecal hydrolysis (Hoekstra et al., 1999). This is important as starch overflow into the cecum is associated with colic, colitis, and laminitis (Garner et al., 1978; Gonçalves et al., 2002; Crawford et al., 2007). Ground grain, however, has more dust than whole grain, and grinding may decrease palatability and increase sorting, leading to feed refusals and nutrient deficiencies (Henry and Morrison, 1923). Pelleting ground grains reduces fines and dust, and it eliminates horses' ability to preferentially sort the feed (Hessel et al., 2009). Because airborne dust particles may increase coughing in horses that suffer from recurrent airway obstruction or inflammatory airway disease, it is often recommended these animals be fed pelleted concentrates with good quality soaked or pelleted forage (Ivester et al., 2014).

Pelleted feed may be particularly beneficial to geriatric horses, as they are often subject to weight loss and deficient body condition resulting from poor dentition and nutrient utilization. Complete pelleted rations contain all required nutrients while reducing duration of mastication (Elia et al., 2010). Pelleted feed also reduces consumption time which may stimulate intake and subsequent weight gain for animals in poor condition. Finally, pelleting feed may increase digestibility of ingredients (Harris et al., 2017).

Pelleted feed is often more economical than whole grains or mashes due to increased bulk density (Earle, 1950; Fairfield et al., 2005), thus decreasing transportation and storage costs. Commercial pelleted feeds are generally designed to meet nutritional requirements of animals during different stages of production and may be designed as a complete diet or used to supplement forages. Thus, feeding commercial pellets often removes the need for owners and managers to develop balanced rations themselves, increasing the likelihood that animal requirements are met. Pelleting allows incorporation of unpalatable feeds or medications that

animals may otherwise refuse. Because fines are removed and recycled for incorporation into other batches (Fairfield et al., 2005), wastage is minimized.

Pellet quality

Modern feed mills have streamlined systems for grinding whole grains, mixing mashes, incorporating liquid ingredients, conditioning mashes, pelleting mashes, and finally cooling pellets. This reduces production costs while yielding structurally sound pellets (Fairfield et al., 2005). Good quality pellets contain little to no dust; withstand forces associated with manufacturing, transportation, and storage (durability); are uniform in length; and have an acceptable appearance without cracks (Payne et al., 2001; Fairfield et al., 2005).

Pellet durability

Pellet durability indices are assigned using tests designed to simulate pellet handling. One of the earliest techniques for measuring pellet durability was use of the Stokes Hardness Tester (Behnke, 2001); however, this test only accounted for compression forces and required the operator to evenly twist the compression screw (Young, 1962). The tumbling box method, developed by Young in 1962, is the current standard for determining pellet durability indices (PDI) as it yields similar results to a model system designed to mimic pellet handling (Young, 1962; Behnke, 2001). Briefly, 500 g clean pellets are placed in a can with or without hex nuts or balls (modified tumbling box) and rotated for 10 min at 50 rpm. Feed is then separated via a sieve. Final weight of pellets retained on the sieve is divided by initial weight prior to simulated handling and multiplied by 100 to calculate PDI (ASAE, 1996; Thomas et al., 1998). Another device commonly used to determine PDI is the Holmen tester which applies pneumatic forces to pellets, forcing them into hard surfaces and right angles (Thomas et al., 1998; Behnke, 2001; Salas-Bringas et al., 2007). While Holmen testers consistently yield fewer intact pellets than

tumble box methods, as long as a consistent testing method is implemented, operators can evaluate and compare pelleting procedures (Fairfield et al., 2005; Fahrenholz, 2012). Regardless of test utilized, most feed mills have adopted 80% to 90% PDI as the acceptable standard (California Pellet Mill Co., 2016).

Factors affecting durability

Understanding factors that contribute to greater PDI is essential to the formation of good quality pellets. Reimer (1992) partitioned the effect of various factors on pellet quality: diet formulation (40%), heat conditioning (20%), particle size (20%), die specifications (15%), and cooling and drying (5%). Moisture added during heat conditioning is limited by the amount of liquid in mashes. Therefore, diet formulation may be considered by some to account for 60% of pellet quality (Fairfield et al., 2005). For the purposes of this review, only diet formulation factors will be evaluated.

Chemical properties of feed ingredients are unique and impact pellet quality; therefore, these properties must be evaluated continuously and pelleting conditions adjusted accordingly (Fairfield et al., 2005). Starch content is one of the most influential properties affecting PDI. When moisture and heat are applied to starch during steam conditioning, moisture penetrates starch molecules and causes gelatinization which binds molecules together upon cooling (Fairfield et al., 2005). In a pellet formulated to contain 40% starch, Wood (1987) reported replacing native starch with pre-gelatinized starch increased PDI and concluded greater inclusion of gelatinized starch may further improve PDI. Mashes which are steam conditioned for longer periods will have greater proportions of gelatinized starch compared to mashes with limited exposure to steam conditioning. Increasing exposure of mashes to steam conditioning, however, may not be practical in industry due to greater production costs; therefore, Thomas and Van der

Poel (1996) recommended steam conditioning long enough to gelatinize only the outer portion of feed particles, thus obtaining acceptable PDI while managing production costs.

Adding pure cellulose at 5% enhances pellet durability without much impact on nutrient content (Buchanan and Moritz, 2009). Equine rations typically contain fibrous feeds such as oat hulls, soybean hulls, alfalfa, and beet pulp. Interestingly, Buchanan and Moritz (2009) reported decreased PDI when increasing fiber, supplied via oat hulls, from 0% to 4%. The authors noted the majority of fines consisted of hulls and thus attributed reduced PDI to grinding size, rather than inclusion of oat hulls (Buchanan and Moritz, 2009). Large feed particles may lead to weak points in pellets where fractures occur, decreasing PDI. Fairfield et al. (2005) also reported poor pelletability of oat hulls, while other fibrous ingredients, such as alfalfa, had moderate pelletability. It has been suggested that poor pelletability of oats may be due to natural abrasiveness and limited interaction with other ingredients (Thomas et al., 1998; Acedo-Rico et al., 2010; California Pellet Mill Co., 2016). Therefore, the texture of fibrous ingredients is believed to impact pellet quality (Fairfield et al., 2005).

Denaturing of complex proteins during heat conditioning also impacts pellet quality. Upon cooling, denatured proteins interact with other molecules, forming new bonds and increasing PDI (Thomas et al., 1998; Buchanan et al., 2010). Buchanan and Mortiz (2009) reported a 7.5% increase in PDI when including 4% soy protein isolate. Others found pellet quality of pure corn pellets was increased with inclusion of soybean meal (Briggs et al., 1999). Pellets formulated to contain approximately 37.9% protein maintained PDI greater than 91% (Tidwell et al., 2017). Ingredients rich in protein, however, are expensive, and, therefore, increasing protein content of pellets beyond animals' nutritional needs is not economical.

There have been few reports on the impact of the interaction of protein, fiber, and starch on pellet quality (Thomas et al., 1998). Buchanan and Moritz (2009) reported effects of fiber and protein each on pellet durability, but they found no evidence of an interaction between the two. Conversely, Tabil et al. (1997) reported inclusion of collagen protein improved PDI of pellets made from poor-quality alfalfa with a low nitrogen fraction; however, when medium or high-quality alfalfa containing greater nitrogen fractions was used, collagen protein had no impact on PDI. This may indicate a protein x forage quality interaction. Higher-quality forages have more soluble CHO and less structural CHO compared to poor-quality forage, but they also have more protein. Therefore, it may be argued total protein content of the pellet formulation is of greater importance than the interaction of protein and forage. Kannadhason et al. (2011) explored the effect of varying protein concentration, starch source, and dried distiller's grains on PDI. The authors reported greater PDI when increasing protein from 28% to 32% in pellets containing cassava and potato starches; however, PDI was reduced in pellets containing corn starch when protein content was increased (Kannadhason et al., 2011). The interaction between starch and protein is dependent on processing of the individual components. Denatured proteins and native starches have limited ability to interact with other components of the pellet, resulting in weaker PDI, whereas inclusion of raw proteins and pre-gelatinized starches will increase PDI (Wood, 1987).

Moisture is considered both a feed and manufacturing factor because it includes water added during conditioning; innate moisture of basic feed ingredients; plus fats, oils, molasses, and flavoring agents (Behnke, 2001). For clarity, however, throughout this review moisture will refer to water or steam added to mashes. Mortiz et al. (2001) compared the effects of low moisture (7%) and high moisture (15%) on pelleting and reported greater mash moisture

increased PDI a minimum of 20%. Authors, however, cautioned > 15% moisture may lead to nutrient dilution. Buchanan and Moritz (2009) reported greater PDI with an additional 4% moisture in mashes, but there was a reduced production rate likely due to excessive moisture, as mash moisture was 18% before moisture was added.

Effects on pellet durability from inclusion of fats and oils are less obvious than that of moisture. Abadi et al. (2019) reported increased PDI when adding 1.5% crude fat powder, while inclusion of 1.5% soy oil did not alter PDI from control. Increasing either fat product to 3% led to reduced PDI (Abadi et al., 2019). Fats may encapsulate starch molecules and thus inhibit gelatinization, reducing friction between particles and decreasing pellet durability (Thomas et al., 1998; Payne et al., 2001; Muramatsu et al., 2015). Oils are often added to lubricate machinery, thus increasing production rate; however, attention must be given to amount of oil added and total liquid in the mash to avoid roll slippage, die plugging and reduced PDI (Gehring, 2009; Abadi et al., 2019).

Mukodiningsih et al. (2010) reported 10% molasses added to corn fodder pellets increased PDI compared to pellets with 5% molasses. This is a result of the thick viscosity of molasses which draws loose particles closer together and subsequently increases pellet integrity (Thomas et al., 1998; Abadi et al., 2019). Due to ingredient-dependent absorbability and moisture content of molasses, however, greater inclusion rates may introduce too much liquid and weaken pellets or plug the roll die (Fairfield et al., 2005; California Pellet Mill Co., 2016). Čolović et al. (2011) reported 9% molasses in wheat pellets led to reduced PDI and thus recommended inclusion rates of 3% to 6% molasses, depending upon the fraction of wheat middlings present in pellets. Molasses inclusion in commercial diets typically ranges from 0% to

10%, depending on total liquid in the formulation (Thomas et al., 1998; Mukodiningsih et al., 2010).

Moisture content of mashes is dependent upon ingredient formulation. Moisture within the mash will dictate length of steam conditioning and each mash will need to be evaluated to determine conditioning settings. Increasing moisture content in mashes may increase PDI; however, exceeding 22% moisture in mashes may lead to weaker pellets and result in complications when pelleting (Moritz et al., 2001; Buchanan and Moritz, 2009). The target moisture of mashes following steam conditioning is < 17% to avoid complications during production (Behnke, 2001; Fahrenholz, 2012; Bortone, 2014). Additional moisture will be added to mashes during steam conditioning; therefore, it is generally recommended only 5% moisture be added to mashes prior to pelleting (Moorhead and Huff, 2005). Adding > 5% moisture may lead to roll slippage and plugging of the roll die, thus damaging equipment and slowing rate of production. Inclusion of fats and oils should be limited to 1% in mashes before pelleting to maintain PDI (Payne et al., 2001). Up to 6% additional oil may be sprayed onto the surface of hot pellets at the pellet die; however, palatability may be affected (Payne et al., 2001; Moorhead and Huff, 2005).

Palatability of equine rations

Many factors affect palatability and ingestion of feed by horses, including taste, odor, texture, and ease of prehension (van den Berg et al., 2016a). Interactions between these factors, however, are largely unknown, with most researchers evaluating the effect of taste and odor simultaneously as it is often difficult and impractical to separate these. Randall et al. (1978) demonstrated horses can differentiate between sweet, salty, sour, and bitter flavors. Horses have preference for sweet water solutions and only partially accept salty, sour, and bitter solutions. It

has been suggested these taste preferences serve a practical purpose, as toxic plants generally have a bitter taste while nutrient dense, easily digestible plants contain more non-structural carbohydrates and are generally sweeter (Houpt et al., 1990; Hill, 2007).

Palatability of forages

Relatively little is known concerning palatability of feedstuffs in the equine despite man's long history of feeding horses. It has been documented horses prefer legumes, such as alfalfa hay, over grass hays (Crozier et al., 1997; LaCasha et al., 1999; McCown et al., 2012). LaCasha et al. (1999) reported increased consumption of cool-season Mutua prairie grass hay (13.5% nitrogen, 62.4% neutral detergent fiber, and 36.1% acid detergent fiber) over warm-season Bermuda grass hay (11.3% nitrogen, 78.3% neutral detergent fiber, and 40.0% acid detergent fiber). McCown et al. (2012) also demonstrated horses prefer cool-season grass hay (timothy; 7.6% nitrogen, 61.7% neutral detergent fiber, and 42.9% acid detergent fiber) over warm-season grass hay (teff; 8.9% nitrogen, 66.0% neutral detergent fiber, and 38.0% acid detergent fiber); however, authors reported similar dry matter intake (DMI) when offering only 1 type of hay. They demonstrated horses would consume enough of either forage type to meet requirements but preferred the cool-season timothy over the warm-season teff. All horses had previously consumed timothy hay, while teff was novel; therefore, preference may have been affected by familiarity of forages (McCown et al., 2012). Another primary factor theorized to impact forage preference is nutrient composition (Redgate et al., 2014). Legumes are generally more nutrient dense than grass hays, having greater crude protein (CP), minerals, and non-structural CHO and fewer structural CHO than grass hays. Similarly, cool-season grass hays typically are more nutritious than warm-season grass hays (NRC, 2007). van den Berg et al. (2016b) reported

horses preferred a high protein diet with added sweetener compared to a low protein diet with added sweetener, indicating horses may select feed based on protein content.

Palatability of concentrates

Less is understood concerning equine preference of grains, as generally limited quantities are offered and adjustments are made gradually to avoid gastrointestinal upset. Preference for individual grains is not typically a concern, as they are often processed and pelleted with a variety of other ingredients, and horses eat most grains readily even if not mixed or pelleted. While inclusion of molasses increases PDI, it also increases palatability of horse feeds. Alternatively, oils and fats may be applied to improve efficiency of pelleting or to increase energy density of pellets, but they may negatively impact palatability. Lindberg and Karlsson (2001) reported reduced intake when adding 28 g maize oil/kg DM to oat-based pellets and 27 g maize oil/kg DM to sugar beet pulp-based pellets compared to oat-based or sugar beet pulp-based pellets without maize oil. Source of fat, however, may influence palatability. Rich (1980) demonstrated horses prefer corn oil, sunflower oil and peanut oil over a variety of other vegetable oils and animal fats; but a control pellet without oil was not offered. Linseed oil has been used to replace barley at 8% supplementation with no negative impact on palatability (Delobel et al., 2008). The maximum level of fats which may be included in equine concentrates before palatability is affected is 15% to 20% (Hallebeck and Beynen, 2002; Gobesso et al., 2009).

Flavoring agents

The most common flavoring agent used in horse feed is molasses, as it is widely accepted by horses. However, other flavoring agents, including herbs and fruits, have been evaluated. Goodwin et al. (2005b) reported horses preferred concentrates flavored with molasses and mint

compared to a combination of molasses, mint, and herbs. While most horses demonstrate preference for sweet mint, data regarding other herbs are contradictory. Horton et al. (1991) reported increased oat intake of 54% and 93% over unflavored feed when 1,500 and 3,000 mg garlic, respectively, were applied to 1 kg pelleted feed. Goodwin et al. (2005b), however, reported concentrate with added molasses, herbs, mint, and garlic to be the least preferred compared to molasses, molasses combined with herbs, and sweetened corn syrup flavored with carrots, herbs, and mint. Fifteen common and historical flavoring agents were evaluated by Goodwin et al. (2005a) with cherry, cumin, and fenugreek have the greatest rates of consumption and refusal of only 3 flavors: echinacea, nutmeg, and coriander. The remaining 12 flavors were paired for preference tests whereby horses slightly preferred peppermint over fenugreek and carrot over rosemary (Goodwin et al., 2005a). Fenugreek, however, was preferred over all other remaining palatants. Thus, authors concluded fenugreek to be the best overall palatant (Goodwin et al., 2005a). Fruit flavors also have been evaluated as palatants with cherry being the most preferred, followed by apple and citrus, and teaberry (Kennedy et al., 2001). Horses preferred flavored oats compared to plain oats (Kennedy et al., 2001). Bottom (2008) reported horses had strong preference for unflavored pellets compared to pellets treated with orange flavoring, though there were large differences between individual horses. When comparing fruit and herb palatants, Parrot et al. (2017) reported fenugreek to be the most preferred palatant compared to banana, mint, cherry, garlic, apple, and carrot.

Neophobic response in the horse

When first introduced to novel ingredients, horses may initially consume only a minute amount, presumably to determine if there will be any ill effects with consumption. Upon determining feed is safe, horses may slowly increase ingestion. This is referred to as a neophobic

response, or fear of new feed, and is considered a survival tactic utilized by herbivores (Hill, 2007). Supporting this theory, Houpt et al. (1990) reported horses demonstrated feed aversion when offered feed that elicited illness immediately upon consumption; however, feed aversion was not observed if onset of illness was delayed. Neophobia is easily interpreted to indicate unpalatable feed, resulting in owners returning to a familiar feed without allowing sufficient time to acclimate to a new feed. van den Berg et al. (2016b) reported it may take 2 to 3 d before consumption of new diets reach 90% of the feed offered, with acceptance considered at 80% consumption. Conversely, introducing new products to horses may elicit a neophilic response, stimulating intake. Gunkel et al. (2015) reported increased consumption of molasses-based blocks in the first 12 h following initial exposure. It is important to note many horse feeds contain molasses and thus, it will not be a novel flavor to all horses. Goodwin et al. (2002) discovered horses offered a variety of hays will spend less time consuming the hay normally presented to them and will increase time spent consuming novel forages; however, authors did not measure consumption of forage.

Summary

While many factors affect pellet quality of horse feeds, concentrate formulation is of upmost importance. Unfortunately, the impact of interactions between various feed components is not yet fully understood, especially regarding impact of liquid products. Inclusion of liquid flavoring agents in pellets formulated for consumption by horses may stimulate feed intake of unpalatable feed ingredients. Palatability in the horse also is not fully understood; however, preference is typically given to familiar or sweet flavoring agents.

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Chapter 4 - Acceptability of Milne blueberry juice and blueberry puree in horse feed

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Abstract

The objective of this study was to evaluate the impact of liquid blueberry juice (BJ65) or blueberry puree (BP30) used as a binding agent on pellet durability and palatability of a typical equine concentrate when included at 4% of the pellet. Molasses was used as a control. Production data, pellet durability, and moisture content were evaluated in 1 replicate for each treatment. Because moisture content of condition mashes was 17.59% and 18.11% for BJ65 and BP30 treatments, respectively, greater inclusion of blueberry product would likely cause roller slippage and complicate the pelleting process due to increased liquid. Pellet durability met industry standards for all treatments. Pellets were fed in a 3-period crossover study to 9 two-year-old horses to determine the effect of blueberry products on acceptability. All animals were allowed 10 min to consume 1.36 kg at 0700 h and 1700 h each day for 3 d. Consumption time and amount consumed were recorded to calculate intake, intake rate, and intake ratio (IR; $\left(\frac{\boldsymbol{BJ65\ (g\ feed\ consumed)}}{\boldsymbol{Control\ (g\ feed\ consumed) + BJ65\ (g\ feed\ consumed)}}\right)$ and $\left(\frac{\boldsymbol{BP30\ (g\ feed\ consumed)}}{\boldsymbol{Control\ (g\ feed\ consumed) + BP30\ (g\ feed\ consumed)}}\right)$). No horses consumed all pellets within the allotted time, and thus, treatment differences for intake and intake rate were the same. Period tended to impact intake ($P = 0.0909$), with horses consuming less during period 1 than period 3 ($P = 0.0317$), but period had no effect ($P = 0.2881$) on IR. Treatment influenced intake ($P < 0.0001$), with decreased intake of BP30 compared to control and BJ65 ($P \leq 0.0001$). Intake ratio was greater ($P = 0.0075$) for BJ65 than BP30 with IR of 0.5069 and 0.4227, respectively. Because IR of 0.50 indicates equal consumption of treatment pellets compared to control, consumption of BJ65 was no different than control. Thus, BJ65 appears to be more acceptable to horses than BP30 when included in dietary pellets at this rate.

Introduction

Due to perceived health benefits associated with natural products, consumers often prefer inclusion of these products in feeds they purchase for companion animals, including horses. Fruit products have long been recognized for health promoting attributes, such as the presence of phenolic compounds which reduce oxidation by interacting with free radicals in the body (Smith et al., 2000; Faria et al., 2005). There is, however, little published research evaluating the palatability of fruit products to horses. Goodwin et al. (2005a) reported acceptance of herbs, spices, and some fruits, including banana and cherry as flavoring agents by horses; however, to our knowledge, there is no published literature reporting the acceptability of blueberry products in equine rations. Blueberry products are commercially available for feed manufacturing and have been incorporated into canine and feline kibble as a source of antioxidants (Dunlap et al., 2006). This project was designed to test pellet quality and acceptability when blueberry products were used as binding agents in a typical feed pellet for horses.

Materials and methods

Animals

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. Nine 2-year-old quarter horses (4 geldings and 5 mares) with an average body weight (BW) of 443.42 kg ± 73.68 kg were used. Horses were housed in randomly assigned stalls (3.05 x 3.66 m) bedded with pine shavings in a single barn for a 3-d acclimation period prior to initiation of the study. Weather permitting, all horses received daily turnout in a dry lot pen for free exercise and socialization throughout the trial. Horse 7 was injured during the free exercise period on the final day of period 2. Following recommendations by the attending

veterinarian, this horse was stalled for the remainder of the experiment. Horse 6 was stalled adjacent to horse 7 during turnout time to prevent separation anxiety in horse 7.

Experimental design and treatments

This was a 3-period crossover study where each period was 3 d in length. Treatments consisted of pelleted feed with 4% molasses (control), Milne Blueberry Juice Concentrate 65 Brix (BJ65), or Milne Blueberry Puree Concentrate 30 Brix (BP30; Milne Fruit Products, Inc., Prosser, WA; Table 4.1). Horses were assigned to 1 of 3 treatment sequences (Table 4.2). After each 3-d period, horses were switched to the next treatment and the process was repeated until all horses had received all treatments.

Throughout each period, 1.36 kg (as-fed) of the designated concentrates were offered for 10 min at 0700 h and 1700 h. Pellets only differed by flavoring agent and were similar in nutritional content (Table 4.3). Horses were offered *ad libitum* access to water throughout each period. Alfalfa hay was also offered *ad libitum* except during the 10-min periods each day when concentrates were offered.

Pelleting

All pellets were manufactured at Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS) in accordance with Current Good Manufacturing Practices (CGMPs). All ingredients were weighed on certified scales with lot numbers recorded and amounts verified. Corn was ground to approximately 600 μm utilizing a 3-high roller mill (924; RMS Roller-Grinder, Harrisburg, SD). All dry ingredients (ground corn, wheat middlings, soybean hulls, alfalfa meal, and soybean meal) were initially added to a Davis Paddle Mixer (S-3; H. C. Davis Sons MFG. Co., Inc, Bonner Springs, KS) and mixed for 60 s. Soybean oil and either molasses (control), Milne (Milne Fruit Products, Inc., Prosser, WA) BJ65, or Milne BP30

were then heated to 57.22°C and sprayed onto dry ingredients. All ingredients were mixed for an additional 360 s to ensure even distribution of liquid ingredients.

Mashes underwent steam conditioning at 79.44°C for 30 s at 25 pounds per square inch (PSI; 254 mm x 1397 mm Wenger twin shaft pre-conditioner, Model 150; Wegner Manufacturing Inc., Sabetha, KS). Mashes were pelleted in a 1-ton pellet mill (1012-2 HD Master Model; California Pellet Mill Co., Crawfordsville, IN) with a 4.7625 mm x 31.75 mm pellet die. Samples were collected upon discharge from the mixer (mash), following steam conditioning (condition mash), at the pellet die (hot pellet), and after cooling (cool pellet) to determine moisture content. Additional samples were collected at the pellet die and following pellet cooling to determine durability according to the 60 s Holmen test and the standard and modified (three 6.35-mm hex nuts) tumble box method (ASAE, 1997). A No. 5 sieve was used to separate fines from pellets before and after durability tests. Percent durability was calculated using the following equation (Stark and Fahrenholz; 2015):

$$\frac{\boldsymbol{g\ pellets\ after\ tumbling}}{\boldsymbol{g\ pellets\ before\ tumbling}}\ \boldsymbol{x\ 100}$$

Preference assessment

Stop watches were used to manually measure consumption time for each horse at each meal. After 10 min, unfinished feed was removed and weighed. After consumption of concentrate had been recorded, alfalfa was offered free choice and remaining pellets were offered to the horse to finish consumption of the pelleted meal. Intake was defined as the amount of feed consumed in the 10 min period. Intake rate of each treatment was calculated via the following equation:

$$\frac{\boldsymbol{Amount\ of\ feed\ consumed\ (g)}}{\boldsymbol{Time\ to\ completion\ (min)\ or\ 10\ min}}$$

No horses completely consumed all pellets within the allotted time; therefore, the full 10-min was used to calculate intake rate for all horses at all meals. Intake ratio (IR) was calculated only on d 3 to minimize the confounding effect of neophobia and was calculated via the following equations:

$$\left(\frac{\boldsymbol{BJ65}\ (\boldsymbol{g\ feed\ consumed})}{\boldsymbol{Control}\ (\boldsymbol{g\ feed\ consumed}) + \boldsymbol{BJ65}\ (\boldsymbol{g\ feed\ consumed})}\right) \textit{and}$$

$$\left(\frac{\boldsymbol{BP30}\ (\boldsymbol{g\ feed\ consumed})}{\boldsymbol{Control}\ (\boldsymbol{g\ feed\ consumed}) + \boldsymbol{BP30}\ (\boldsymbol{g\ feed\ consumed})}\right)$$

Statistical analyses

Data collected during pelleting were not subjected to statistical analyses as only 1 sample per treatment was obtained for moisture and durability testing.

Intake data were analyzed using the GLIMMIX procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC) with option of DDFM=KR used for approximating degrees of freedom. Fixed effects of the model included treatment (BP30, BJ65, and control), day (period), and a treatment × day (period) interaction. Random effects of the model included horse, period (1, 2, and 3), and intercept of treatment × horse. Least square means and their standard errors were reported using the PDIFF option. Pairwise comparisons were evaluated via the 2-sided test for non-zero differences at significance level $P < 0.05$. Tendencies were declared when $0.05 < P < 0.10$.

Results

Production data

Data regarding production and pellet durability are presented in Table 4.4. Mash moisture following mixing ranged from 12.36% in control to 13.66% in BP30. Moisture of condition mashes increased to 17.59% for BJ65 and 18.11% for BP30 compared to 15.86% moisture in

control. While hot pellet moisture varied from 14.08% to 17.58% in BP30 and BJ65 pellets, respectively, moisture of cooled pellets ranged from 12.98% in control to 13.49% in BJ65. All pellets achieved greater than 95% pellet durability index (PDI) when using the standard tumble box method. The modified tumble box yielded PDI values between 89.95% and 92.46%. The Holmen test was most destructive to pellets and yielded PDI ranging from 89.31% to 90.95%.

Intake

There was a tendency ($P = 0.10$; Fig. 4.1) for a treatment × day (period) interaction on intake (kg/meal) whereby horses consumed greater ($P \leq 0.03$) quantities of control pellets compared to BP30 pellets during the first 2 d of period 1. Feeding control and BJ65 pellets led to greater ($P \leq 0.02$) consumption compared to BP30 during the first 2 d of period 2. There were no differences between intake of treatments during period 3. Intake was affected by treatment ($P < 0.0001$; Table 4.5) with BP30 decreasing intake of concentrate compared to BJ65 ($P = 0.0001$) and control ($P < 0.0001$). There was no difference ($P = 0.15$) between intake of BJ65 compared to control. There was no main effect ($P = 0.32$) of day (period); however, horses consumed greater quantities of each treatment by d 3 compared to d 1 during periods 1 ($P < 0.0001$) and 2 ($P = 0.02$), with a tendency ($P = 0.09$) to consume greater quantities on d 3 compared to d 1 during period 3. There was a tendency for intake to be affected by period ($P = 0.09$). Horses consumed less ($P = 0.03$) during period 1 compared to period 3 (data not shown).

Because horses failed to consume the 1.36 kg meal in the 10 min allowed, g consumed for each horse was divided by the total time allowed (10 min) to calculate intake rate. Because the same denominator was used in all cases, the data generated for intake rate (g/min) was redundant to the data generated for intake. Thus, intake rate also was affected by treatment ($P < 0.0001$), with a tendency for period ($P = 0.09$) and treatment × day interaction ($P \leq 0.10$). As

only units changed, statistical analyses for intake rate reflected the same differences noted for intake as detailed above.

Intake ratio

There was a main effect ($P = 0.01$) of treatment on IR, with BJ65 having a greater ($P = 0.01$) IR compared to BP30. There was no effect ($P = 0.29$) of period on IR.

Discussion

Production of pellets

The similarities in production rate, condition temperature, and hot pellet temperature between treatments were not surprising as total liquid in mashes were similar. The moisture of condition mash of concentrates containing BJ65 and BP30, however, indicates 4% inclusion is likely the maximum amount of liquid blueberry product that should be added to this mash formulation, as the maximum recommended moisture during pelleting is 17% (Bortone, 2014). Additional product would likely cause complications during feed manufacturing, such as roll slip, thus increasing production costs (Fahrenholz, 2012; Bortone, 2014). Data collected regarding pellet durability using these techniques correspond well with data collected by Fahrenholz (2012) who reported the standard tumble box method yields the greatest pellet durability indices and the 60 s Holmen test yields the lowest pellet durability indices. All pellets met industry standards (10% to 20% fines; California Pellet Mill Co., 2016) as they contained just below 10% fines following all durability tests. Therefore, the production methods utilized in the present study yielded pellets that should withstand forces associated with bagging, transportation, and storage. Heating liquid products for application onto dry ingredients and

temperatures of condition mashes, however, may reduce anti-oxidant capacity of blueberry products.

Consumption

All pellets in the present study were manufactured using the same basal formulation and proximate analyses of nutrients was similar across treatments. Therefore, flavoring should have been the driving factor influencing consumption. Treatment altered acceptability as demonstrated by reduced intake of BP30 compared to BJ65 and control. This is further supported by the fact that IR for BP30 was less than BJ65. Intake ratios of 0.50 represent equal acceptability when compared to the control, while values below 0.50 indicate acceptability was less than control, which was the case with BP30. While inclusion of blueberry products within horse feed has, to our knowledge, not been reported in literature, horses readily consume a variety of flavors (Goodwin et al., 2005b). van den Berg et al. (2016) reported horses preferentially select feed based on nutritive value, followed by preference for sweeter feed, and finally odor plays a role in feed preference. This is in accord with our observations as degrees Brix is a measurement of specific gravity and is used to quantify sugar content of liquids (Caldwell, 2001; Moorhead et al., 2005). As degrees Brix increase, so does sugar content; therefore, BJ65 had greater concentration of sugar than BP30. The degree Brix of beet molasses is 79.5; thus, these horses likely did not differentiate between sweetness of feed with degree Brix 65 and degree Brix 79.5. If any difference between sweetness was detected, perhaps flavoring of BJ65 enhanced intake enough to overcome differences in sweetness between molasses and BJ65.

Horses displayed a neophobic response to the changed concentrate on the first 2 d of periods 1 and 2, depending on treatment offered; however, by d 3 there were no differences in amount of pellets consumed between treatments. Similarly, van den Berg et al. (2016) reported 2

to 3 d are required for horses to overcome neophobic responses enough to consume 90% of feed offered. There were no differences in intake between days within period 3, indicating horses were no longer exhibiting feed aversion. By the final period, all horses had previously received at least 1 treatment containing blueberry product; thus, horses may not have had as strong of a neophobic response to the remaining treatment, resulting in greater (P = 0.0317) consumption during period 3 compared to period 1. Interestingly, horses offered BP30 during period 2 had received BJ65 during period 1 whereas horses which consumed BJ65 during period 2 had previously only consumed control. Therefore, it may take horses longer to accept BP30 compared to BJ65, even if previously exposed to a blueberry product. Even though any feed not consumed within the allotted time was offered back to horses, it is possible temporary removal of feed gradually conditioned horses to increase rate of consumption, resulting in more consistent consumption of treatments during the final period.

Conclusion

Liquid blueberry product can replace 4% molasses in equine feed pellets consisting of 45% wheat middlings and 25% corn without reducing pellet durability. Milne Blueberry Juice Concentrate 65 Brix offers similar palatability to molasses in horse feed. Conversely, horses are less likely to accept Milne Blueberry Puree Concentrate 30 Brix when compared to molasses. This experiment, however, was not designed to assess various inclusion rates, potential negative side effects, or antioxidant activity. Moreover, due to heat associated with the application and pelleting process, antioxidant activity of blueberries may be reduced.

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Table 4.1. Composition of pelleted feed[1]

Ingredient, %	Control[2*]	BJ65[3*]	BP30[4*]
Wheat middlings	45.0	45.0	45.0
Corn	25.0	25.0	25.0
Soybean hulls	15.0	15.0	15.0
Alfalfa meal	10.0	10.0	10.0
Soybean meal, 46% crude protein	4.0	4.0	4.0
Soy oil	1.0	1.0	1.0
Molasses	4.0	--	--
Milne Blueberry Juice 65 Brix	--	4.0	--
Milne Blueberry Puree 30 Brix	--	--	4.0

[1]Offered to horses at a rate of 1.36 kg/meal (as-fed) for 10 min at 0700 h and 1700 h.
[2]Molasses included at 4% of concentrate.
[3]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
[4]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
[*]Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS).

Table 4.2. Treatment assignment and sequences

Horse	Period 1[*]	Period 2[*]	Period 3[*]
1	BP30[1‡]	Control[2‡]	BJ65[3‡]
2	BJ65	BP30	Control
3	BP30	Control	BJ65
4	Control	BJ65	BP30
5	BP30	Control	BJ65
6	Control	BJ65	BP30
7	Control	BJ65	BP30
8	BJ65	BP30	Control
9	BJ65	BP30	Control

[*]Each period was 3 d in length.

[1]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[2]Molasses included at 4% of concentrate.

[3]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[‡]Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS) and offered at a rate of 1.36 kg/meal (as-fed) for 10 min at 0700 h and 1700 h.

Table 4.3. Nutrient composition of dietary concentrate and forage (dry matter basis)[1]

Item	Control[2]*	BJ65[3]*	BP30[4]*	Alfalfa hay[5]
Dry matter, %	87.40	88.40	88.10	92.10
Crude protein, %	16.40	16.20	16.10	17.30
Acid detergent fiber, %	18.40	19.60	19.50	40.10
Neutral detergent fiber, %	33.20	33.10	33.80	19.60
Ether extract, %	--	--	--	1.70
Ash, %	--	--	--	9.20
Calcium, %	--	--	--	1.10
Phosphorus, %	--	--	--	0.31
Digestible energy, Mcal/kg	3.03	3.03	3.02	2.21

[1]Control, BJ65, and BP30 were analyzed via wet chemistry and alfalfa hay was analyzed via forage NIR (Dairy One, Ithaca, NY).

[2]Molasses included at 4% of concentrate.

[3]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[4]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[*]Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS) and offered at a rate of 1.36 kg/meal (as-fed) for 10 min at 0700 h and 1700 h.

[5]Fed *ad libitum* throughout the experiment except during the 10-min periods each day when concentrates were offered.

Table 4.4. Characteristics of pellets through production.

Item	Control[1+]	BJ65[2+]	BP30[3+]
Production rate, kg/min	9.39	8.98	9.43
Condition temperature, °C	80.22	80.44	79.72
Hot pellet temperature, °C	84.00	84.22	85.00
Moisture, %			
Mash	12.36	13.20	13.66
Condition mash	15.86	17.59	18.11
Hot pellet	16.84	17.58	14.08
Cool pellet	12.98	13.49	13.12
Pellet durability, %			
Standard tumble[*]	95.30	95.75	95.97
Modified tumble[‡]	89.95	91.72	92.46
Holmen[‡*]	89.31	90.74	90.95

[1]Molasses included at 4% of concentrate.
[2]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
[3]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
[+]Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS) and offered at a rate of 1.36 kg/meal (as-fed) for 10 min at 0700 h and 1700 h.
[*]Tumble box method.
[‡]Three 6.35-mm hex nuts included in tumble box.
[‡*]60 s run time.

Table 4.5. Effect of blueberry juice (BJ65) and blueberry puree (BP30) on mean intake[*], mean intake ratio of a pelleted feed[ǂ] in 2-year-old Quarter horses[†].

Item	Control[1+]	BJ65[2+]	BP30[3+]	SEM	*P*-value
Intake per meal, kg/meal	1.0004[a]	0.9223[a]	0.6468[b]	0.05174	< 0.0001
Intake ratio[‡]	--	0.5069[a]	0.4227[b]	0.01931	0.0075

[ǂ]Concentrates were fed at a rate of 1.36 kg/meal (as-fed) at 0700 and 1700 h for 10 min.

[†]9 horses were utilized in a 3-period crossover design.

[*]Averaged over 3 d.

[‡]Grams of BJ65 pellet consumed/(g of BJ65 pellet consumed + g of control pellet consumed) or g of BP30 pellet consumed/(g of BP30 pellet consumed + g of control pellet consumed) averaged over the 2 meals consumed on d 3 of each period.

[1]Molasses included at 4% of concentrate.

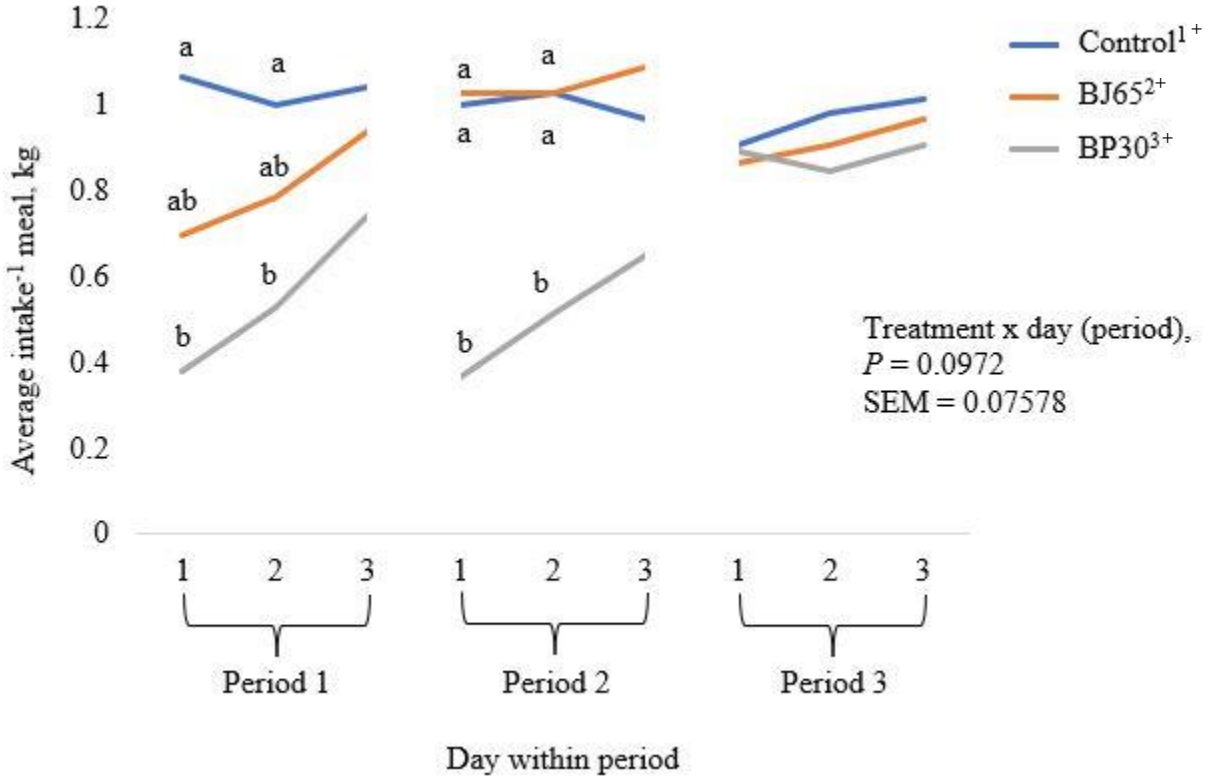
[2]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[3]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.

[+]Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS) and offered at a rate of 1.36 kg/meal (as-fed) for 10 min at 0700 h and 1700 h.

[a,b]Means within a row with a different superscript differ ($P < 0.05$).

Figure 4.1. Effect of blueberry juice (BJ65) and blueberry puree (BP30) on mean intake (kg/meal) of a pelleted feed⸸ in 2-year-old Quarter horses*.



⸸Concentrates were fed at a rate of 1.36 kg/meal (as-fed) at 0700 and 1700 h for 10 min.
*9 horses were utilized in a 3-period crossover design.
[1]Molasses included at 4% of concentrate.
[2]Milne Blueberry Juice Concentrate 65 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
[3]Milne Blueberry Puree Concentrate 30 Brix (Milne Fruit Products Inc., Prosser, WA) included at 4% of concentrate.
+Concentrates were mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center (Kansas State University, Manhattan, KS).
[a,b]Means with a different letter on a given day differ ($P < 0.05$).

Chapter 5 - Literature review: Equine digestion with emphasis on the microbiome of the cecum

Introduction

In forage-fed cattle, rumen degradable protein is a limiting nutrient for maintenance and proliferation of ruminal microorganisms, and, subsequently, microbial fermentation (Köster et al., 1996). Due to pre-cecal digestion of N in the horse, it is even more likely the microbial ecosystem of the equine hindgut is a N-limited environment (Santos et al., 2011). When supplying N to microbial communities, more soluble sources of N are associated with more rapid and complete degradability (Zhao et al., 2022; Hedqvist and Udén, 2006). Additionally, source of N available to microorganisms also should be considered, as several microorganisms, specifically fibrolytic microorganisms, require branch-chained fatty acids (BCFA) as growth factors (Stewart et al., 1997). To facilitate maximum growth of microorganisms and maximize microbial efficiency, however, adequate supply of N, growth factors (BCFA), and energy must be supplied simultaneously.

Carbohydrates (CHO) serve as the primary source of energy to the horse and microorganisms in the equine hindgut. As hindgut fermenters, non-structural carbohydrates (nsCHO) are digested and absorbed in the small intestine (Chesson and Forsberg, 1997). Any sCHO that exceeds the digestive and absorptive capacity of the small intestine then enters the cecum and is subjected to microbial fermentation. Large quantities of nsCHO entering the cecal environment, however, can result in shifts in microbial populations, favoring proliferation of starch-utilizing (amylolytic) microorganisms and limiting proliferation of fiber-utilizing (fibrolytic) microorganisms (Warzecha et al., 2017). To attenuate drastic shifts in microbial populations and negative effects of large quantities of dietary starch entering the cecum, it is

recommended to offer horses a maximum of 2 to 4 g starch·kg BW^{-1}·$meal^{-1}$ (Potter et al., 1992). The effect of feeding starch at this rate on the equine cecal microbiome, however, is not fully understood.

Advancements in sequencing technology, specifically next generation sequencing (NGS), have expanded capabilities of researchers who study microbial populations. Next generation sequencing does not require complicated cloning of fragments of deoxyribose nucleic acid (DNA), but rather amplification of fragments of DNA via polymerase chain reaction (PCR), thus reducing time required to obtain necessary quantities of DNA for sequencing (Slatko et al., 2018). Expanded databases have facilitated rapid identification of microbial species (Schloss et al., 2009). The role of identified microorganisms in metabolism, however, is necessary to determine impact of diets, medication, or stress on microbial ecosystems.

The equine gastrointestinal tract and digestion of protein and carbohydrates will be covered in this review, focusing on fermentation of protein and carbohydrates by equine cecal microorganisms. The effect of dietary starch on the equine cecal microbiota will also be evaluated. Lastly, the process of next generation sequencing will be discussed, with emphasis on 16S rDNA sequencing.

Gastrointestinal physiology of the horse

Introduction

The natural diet of horses consists of poor-quality forage, consumed almost continuously throughout the day (Bott et al., 2013). Fibrous feedstuffs are first subjected to enzymatic digestion in the stomach and small intestine, where lipids, protein, and starch are digested and absorbed. This is followed by extensive microbial fermentation of fiber throughout the hindgut (Santos et al., 2011), yielding volatile fatty acids (VFA) that provide energy to the horse.

Without microbial fermentation, this energy would otherwise be unavailable to the horse as mammalian enzymes cannot degrade fiber (Bergman, 1990). Efficiency of degradation of fiber is dependent upon rate of passage (ROP), extent of pre-cecal digestion, and dietary composition.

Physiology of the equine foregut

Like that of other herbivores, saliva produced by horses contains limited enzymatic activity, with less than 5 U/L salivary amylase activity (Boehlke et al., 2015; Contreras-Aguilar et al., 2018). Therefore, the contribution of saliva to degradation of feed is negligible. Instead, the main purpose of salivary production in the equine is to lubricate feed as it travels through the esophagus and to buffer gastric contents (Alexander, 1966).

The stomach of an adult horse comprises approximately 8% of the total volume of the gastrointestinal tract (Cuncha, 1991; Di Filippo et al., 2016) and contains both squamous and glandular regions, which are demarcated by the margo plicatus (Banse and Andrews, 2019). The squamous portion is non-glandular and is located in the upper third of the stomach, near the cardiac sphincter. Gastric pH within the non-glandular region is approximately 4.0, which is greater than the pH found in other regions of the equine stomach (Al Jassim, 2006). The glandular mucosa contains 3 regions: cardiac, fundic, and pyloric. Within the glandular region of the stomach, the cardiac region is most proximal to the margo plicatus and consists of mucus secreting glands which may aid in modulating gastric pH (Merritt, 1999; Verkola, 2014). The fundic region comprises the largest portion of the glandular mucosa and contains parietal and chief cells which secrete hydrochloric acid (HCl) and pepsinogen, respectively (Merritt, 1999). The fundic region also contains enterochromaffin-like cells which secrete histamine and serotonin, hormones that reduce secretion of gastric acid and gastric blood flow (Merritt, 1999; Rabuffo et al., 2009). The pyloric region, sometimes referred to as the antrum, is comprised of

enterochromaffin-like cells, as well as G cells and D cells, which secrete gastrin and somatostatin, respectively (Merritt, 1999; Rabuffo et al., 2009). Gastrin is a hormone that stimulates production and release of gastric acid (Wilson et al., 2007). Meanwhile, secretion of somatostatin suppresses secretion of gastrin (Merritt, 1999). The pH of chyme within the glandular region of the equine stomach is typically between 2.0 and 2.5, depending on diet (Nadeau et al., 2000; Al Jassim, 2006). Despite this acidic pH, the equine stomach contains a population of viable microorganisms. Perkins et al. (2012) reported various species of *Lactobacilli*, *Streptococcus* sp., *Sacrina* sp., *Eubacterium* sp., *Acrinobacillus* sp., *Moraxella* sp., *Acinetobacter* sp., and *Veillonella* sp. present on the lining of the stomach. While there was no effect of sampling location (non-glandular, glandular, and antral) on relative abundance of microorganisms identified (Perkins et al., 2012), microbial populations within the equine stomach vary with dietary composition, health of the horse, gastric pH, and integrity of the gastric lumen (Varloud et al., 2007; Perkins et al., 2012).

Despite this microbial presence and continuous gastric secretions, digestion of feed within the equine stomach is limited due to rapid ROP. Rate of passage through the stomach varies from 2 to 6 h, with an average ROP through both the stomach and small intestine of 5 h (Van Weyenberg et al., 2006). Mean retention time (MRT) is dependent upon particle size, processing, dietary composition, meal size, water consumption, saliva production, and animal characteristics including weight, level of exercise, and stage of production (Van Weyenberg et al., 2006). Métayer et al. (2004) evaluated the impact of starch content and meal size on the time required for half-emptying of the equine stomach. Horses consuming 2.47 Kcal/g of a low-starch diet had more rapid gastric half-emptying than horses consuming 3.26 Kcal/g of a high-starch diet, and smaller meals led to faster gastric half-emptying compared to larger meals (Métayer et

al., 2004). There is also evidence the liquid phase has a reduced MRT compared to the particulate phase of digesta (Sojka and Cantwell, 1988). Despite a rapid ROP, the equine stomach is never empty, as peristaltic contractions are initiated upon consumption of feed and decrease when consumption ceases (Van Weyenberg et al., 2006).

Feed enters the first section of the equine small intestine through the pyloric sphincter. The small intestine comprises approximately 30% of the total volume of the equine gastrointestinal tract and is the primary site for digestion and absorption of nutrients in the equine foregut (Cuncha, 1991). The small intestine is separated into 3 sections: the duodenum, the jejunum, and the ileum. The duodenum is the most proximal section of the small intestine, extending approximately 1 m from the pyloric sphincter to the duodenojejunal flexure (Epstein and Fehr, 2013). The jejunum extends 17 to 28 m, depending upon size of the adult horse, and is loosely attached to the abdomen via the mesojejunum (Freeman, 2006). The ileum is the most caudal segment of the equine small intestine and is approximately 0.7 m long, terminating at the ileocecal fold (Merritt and Julliand, 2013). Visually, it is difficult to differentiate segments of the small intestine; however, the types of glands and secretions vary throughout the small intestine and can aid in distinguishing the 3 regions.

Hepatic and biliary secretions enter the duodenum primarily through small ducts located near the pyloric sphincter. While hepatic and biliary secretions are continuous, acidic chyme entering the duodenum stimulates a marginal increase in these secretions (Kararli, 1995). Various enzymes are present in hepatic secretions, including trypsin, chymotrypsin, elastase, amylase, and lipase (Lorenzo-Figueras et al., 2007). Trypsin, chymotrypsin, and elastase degrade proteins. Soluble carbohydrates are degraded via amylase. Lipase and bile salts degrade lipids. Bicarbonate, supplied via bile, increases pH of digesta to between 5.6 and 6.6, depending on diet

(Mackie and Wilkins, 1988; de Fombelle et al., 2003; Al Jassim, 2006). This increase in pH activates enzymes present in hepatic secretions and facilitates digestion and absorption of nutrients. Brunner's glands, or duodenal glands, secrete alkaline mucus which protects the mucosa, or lining, of the intestinal lumen (Florey and Harding, 1935; Krause and Leeson, 1967). In most mammals, these glands are restricted to the duodenum; however, in horses, Brunner's glands are also found in the jejunum (Grossman, 1958). As digesta moves through the small intestine, secretions from Brunner's glands further increase pH of digesta to 6.0 to 7.2 in the jejunum and 6.5 to 7.5 in the ileum (Mackie and Wilkins, 1988; de Fombelle et al., 2003; Al Jassim, 2006). The ileum contains unique gut-associated lymphoid tissue, known as Peyer's patches, which play an important role in protection against pathogenic microorganisms (Jung et al., 2010; Jennings and Premanandan, 2017).

The inner mucosal layer throughout the small intestine, regardless of section, has similar anatomy. The small intestinal mucosa is complex and covered in villi, or small finger-like projections that increase luminal surface area. Each villus is covered in a layer of goblet cells and enterocytes which secrete mucus and absorb nutrients, respectively (Gonzalez et al., 2015). Crypts of Lieberkühn, located between villi, contain epithelial stem cells, Paneth cells, and enteroendocrine cells. Epithelial stem cells replace cells of the mucosal lining every 5 to 7 d and differentiate into various intestinal cells, including Paneth and enteroendocrine cells. Paneth cells produce antimicrobials, and enteroendocrine cells produce hormones (Takehana et al., 1998; Gonzalez et al., 2015; Stewart et al., 2018). Each enterocyte has a brush border of microvilli that contain digestive hydrolases and carrier proteins for nutrient absorption, further increasing digestive and absorptive capacity of the small intestine. The integrity of the luminal surface of the small intestine is dependent upon tight junctions between enterocytes, which modulate

transport of fluid, electrolytes, and solutes (Suzuki, 2013). Larger molecules such as antigens and bacteria cannot cross through the border of healthy tight junctions (Suzuki, 2013).

Extent of digestion and absorption in the small intestine is largely dependent upon ROP of digesta. Due to challenges associated with sampling digesta, transit time through the small intestine is not typically recorded; instead, ROP throughout the equine foregut, consisting of the stomach and small intestine, has been more thoroughly documented. From the time of ingestion, 3 to 6 h is required for digesta to move through the small intestine (Drogoul et al., 2000; de Fombelle et al., 2003). Accounting for MRT of feed in the stomach, passage through the small intestine may occur in as little as 1 h (Van Weyenberg, 2006). There is evidence that ROP varies based on feeding status of the animal and specific segment of the small intestine. Farooq et al. (2016) reported 5 to 6 contractions/min in the duodenum of fed mules; however, rate was reduced to 2 to 3 contractions/min and 0 to 1 contractions/min when animals were fasted for 12 and 24 h, respectively. The jejunum appeared to be the most motile, with 5 to 15 contractions/min in fed animals but was reduced to 4 contractions/min when mules were fasted (Farooq et al., 2016). Thus, ROP is more rapid when animals are fed vs. fasted and through the jejunum vs. the duodenum.

Physiology of the equine hindgut

Digesta leaving the small intestine enters the cecum through the ileocecal junction. The equine cecum is a sacculated blind pouch with a volumetric capacity of approximately 30 L and is 1 to 2 m long, comprising 15% of the total volume of the equine digestive tract (Cuncha, 1991; Al Jassim and Andrews, 2009). Microorganisms present in the equine cecum and other components of the large intestine produce enzymes that degrade structural carbohydrates, yielding VFA (Julliand et al., 1999). These VFA’s serve as an energy source for the horse. In

addition to structural carbohydrates, cecal microorganisms can ferment soluble carbohydrates, protein, intermediary products of fermentation, and remove fatty acids from alcohol backbones of lipids. The relative proportions of different functional groups of equine cecal microorganisms are dependent upon dietary composition (Medina et al., 2002; Warzecha et al., 2017). The extent of fermentation, particularly fermentation of structural carbohydrates, in the equine cecum is dependent upon several factors including cecal pH, dietary composition, and ROP.

In healthy animals, cecal pH ranges from approximately 6.2 to 7.1, depending on diet and time of sampling (Medina et al., 2002; de Fombelle et al., 2003; Venable et al., 2017; Warzecha et al., 2017). A reduction in cecal pH occurs approximately 3 to 6 h following consumption of grain (Medina et al., 2002; Jones, 2015; Reeg, 2015; Venable et al., 2017; Warzecha et al., 2017). Post-prandial reduction in pH corresponds with feed entering the cecum and subsequent microbial fermentation and production of VFA (Kristoffersen et al., 2016; Warzecha et al., 2017). When cecal pH is < 6, cellulolytic enzymatic activity may be reduced, limiting fermentation of fiber (Morrison, 1976; Lowe et al., 1987; Hu et al., 2004; Sung et al., 2006). Cecal pH, however, is rarely < 6, unless large amounts of starch are consumed (Medina et al., 2002; Venable et al., 2017; Warzecha et al., 2017). When feeding forage-only diets, there may not be a post-prandial reduction in cecal pH (Kristoffersen et al., 2016; Warzecha et al., 2017).

Another important role of the equine cecum is fluid absorption, which is also dependent upon diet. Ponies fed cellulose-based pellets had less fluid absorbed in the cecum than ponies fed a typical hay and concentrate ration (Argenzio et al., 1974b). Interestingly, the equine cecum lacks villi (Kotzé and Soley, 1990); however, columnar cells that comprise the cecal mucosa have microvilli, contributing to absorption of water and nutrients in the cecum (Blikslager and Gonzalez, 2018). The equine cecal mucosa also contains crypts of Lieberkühn, enterocytes, and

large amounts goblet cells (Kotzé and Soley, 1990; Jennings and Premanandan, 2017). Sacculations in the cecum, known as haustra, serve to mix digesta and slow ROP. There are 4 bands of smooth muscle and connective tissue, or teniae, which support the cecum (Jerbi et al., 2014). Teniae are richly innervated and contribute to peristalsis, pushing digesta through the ceco-colic flexure (Burns, 1992; Burns, 1996). The average MRT of digesta in the cecum when feeding hay or silage is 2.9 h (Miyaji et al., 2008). Rate of passage through the equine cecum, however, is dependent upon fraction of digesta: liquid phase or particle phase. Drogoul et al. (2000) reported greater MRT of particle phase (4.9 h) than liquid phase (2.0 h) when feeding horses chopped hay. When feeding ground hay, however, MRT of the particle phase (1.9 h) and liquid phase (1.6 h) was similar (Drogoul et al., 2000). Thus, retention time of feed particles in the cecum depends on particle size. Argenzio et al. (1974b) evaluated MRT of various particle sizes of feed throughout the gastrointestinal tract of Shetland ponies. Forty-eight hours following administration of markers into the ceca of Shetland ponies, approximately 10% of the liquid, 2 mm, and 1 cm markers remained in the cecum, while approximately 20% of the 2 cm marker remained in the cecum (Argenzio et al., 1974b). Furthermore, based on calculated retention curves, 10% of the 2 cm, 8% of the 2 mm, and 5% of the 1 cm marked feed would remain in the cecum 3 d post cecal dosage. Smaller quantities of 2 cm and 2 mm markers were estimated to be retained in the cecum for up to 10 d, while 100% of the 1 cm marked feed was projected to be removed 8 d post-dosing (Argenzio et al., 1974b). It is interesting that 1 cm marked feed was projected to have the most rapid ROP based on retention curves, as it was not the longest or shortest particle size. This may be due to error associated with the equations used to calculate retention curves. The equations utilized for retention curves were expected to have an error of ± 5%; however, variability between horses increased error to ± 25%.

Digesta leaves the cecum and enters the large colon through the ceco-colic orifice, which is positioned just distal to the ileocecal junction (Rakestraw and Hardy, 2006). The large colon comprises approximately 38% of the total volume of the equine digestive tract and fills a similar physiological role as the cecum, containing a large population of anaerobic microorganisms that degrade fibrous dietary components and yield VFA (Cuncha, 1991). There are 4 sections of the ascending large colon: right ventral colon (RVC), left ventral colon (LVC), left dorsal colon (LDC), and right dorsal colon (RDC).

Similar to the cecum, the mucosa of all sections of the equine large colon contains mucus-secreting goblet cells, with RDC having a denser concentration of goblet cells compared to RVC (Lopes and Pfeiffer, 2000). Enteroendocrine cells are present in cytoplasmic granules positioned near the basement membrane of the mucosa throughout the large intestine. Enteroendocrine cells secrete a variety of hormones and are vital to normal gut function (Daly et al., 2012; Gonzalez et al., 2015). The mucosa throughout the large intestine lacks villi, but enterocytes line glands in the colonic mucosa and are responsible for absorption of VFA and fluid (Lopes and Pfeiffer, 2000). Additionally, similar to the cecum, there are microvilli which contribute to integrity of the luminal lining of the equine large colon (Grosche et al., 2011).

The pH of different sections of the equine large colon may vary slightly. de Fombelle et al. (2003) reported pH of digesta in the RVC, LVC, LDC, and RDC to be 6.2, 6.6, 6.6, and 6.2, respectively, when feeding horses a high-fiber or a high-starch diet (de Fombelle et al., 2003). While pH is relatively consistent, MRT of digesta differs by section of the large colon. Miyaji et al. (2008) fed first-cutting timothy hay or haylage to Thoroughbred horses. Average MRT of digesta was 3.1 h, 5.9 h, 1.0 h, and 4.0 h in the RVC, LVC, LDC, and RDC, respectively (Miyaji et al., 2008). Differences in MRT between sections of large colon is largely attributed to orifices,

specifically the ventral-dorsal and dorsal-small colonic junctions (Argenzio et al., 1974b). Retropulsive peristalsis, which originates at the pelvic flexure between the LVC and LDC, also slows caudal propulsion of digesta, specifically large feed particles, in the horse (Sellers et al., 1979; Sellers and Lowe, 1986). As a result, greater proportions of digesta are maintained in the LVC compared to other sections of the large colon (Miyaji et al., 2008). Within the RDC, particles ≤ 2 mm and liquid are retained for longer periods of time compared to larger feed particles (Drogoul et al., 2000). This retention may be a result of retropulsive peristalsis and likely contributes to fluid absorption (Hummel et al., 2018). The transition from RDC to transverse colon is marked by a slight narrowing of the diameter of the intestine (Merritt and Julliand, 2013). The transverse colon is responsible for compressing digesta and absorbing liquid (Drogoul et al., 2000), but information regarding the equine transverse colon is limited.

As the transverse colon curves caudally, it transitions to the small colon just under the left kidney, with the only delineation between the 2 sections being a narrowing in the diameter of the lumen. The small colon is 3 to 4 m in length and comprises approximately 9% of the total volume of the equine digestive tract (Cuncha et al., 1991). Argenzio et al. (1974a) measured no net production of VFA (production of VFA minus absorption of VFA) in the small colon; however, microbial fermentation of fiber occurs at similar or greater rates in the small colon than in the cecum, depending on dietary composition (Varloud et al., 2004). Another primary function of the small colon is absorption of water and formation of fecal balls (Argenzio et al., 1974b). Mean retention time of digesta in the small colon is approximately 4 h (Miyaji et al., 2008), which facilitates microbial fermentation and absorption of water and VFA. The small colon terminates at the rectum.

The rectum begins at the pelvic inlet and is approximately 30 cm long (Sisson, 1975). The role of the rectum is to temporarily store fecal matter. As fecal matter enters the rectum, internal rectal pressure increases until the retrosphincteric reflex is initiated (Herdt, 2019). The retrosphincteric reflex is a relaxation of the internal anal sphincter followed by peristalsis, resulting in defecation (Herdt, 2019). Total fecal output will vary greatly depending on weight of the horse, as larger horses have a greater capacity for feed intake, and, thus, body weight is generally used to normalize fecal output data. Williams et al. (2015) reported an average fecal output of 4.62 kg/100 kg BW/d, or between 23 and 36 kg fecal matter/d, for horses weighing between 500 and 800 kg. In addition to body weight, health and hydration status of the animal, dietary composition, and extent of digestion throughout the gastrointestinal tract also impact fecal output (Williams et al., 2015). In total, it generally takes approximately 31 to 43 h for feed to traverse from the mouth to the anus of the horse, depending on diet and exercise (Pagan et al., 1998).

Protein digestion in the equine

Requirement of protein in the equine diet

The National Research Council (NRC) recommends mature horses at maintenance consume 1.26 g crude protein (CP)·kg BW^{-1}·day^{-1} (NRC, 2007). Amount of protein recommended, however, depends on stage of life and activity level of an individual horse (NRC, 2007). While CP recommendations serve as useful guidelines, horses also have requirements for specific amino acids (AA; Gibbs and Potter, 2002). There are more than 300 AA found in nature; however, only 20 AA are utilized to build muscle. These 20 AA have been researched extensively and are classified as essential, non-essential, or conditionally essential from a nutritional perspective (Wu, 2009).

Essential AA are those that must be supplied through the diet as their carbon skeletons cannot be synthesized in the body (Wu, 2009). There are 9 essential AA for mammals: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Wu, 2009; Urschel and Lawrence, 2013). The NRC, however, only provides a dietary requirement (36 mg·kg BW^{-1}·day^{-1} for inactive mature adult horses in maintenance) for lysine, the first limiting AA for protein synthesis in the horse (NRC, 2007). Lack of information regarding requirements of other essential AA for horses is due to a variety of factors that are confounded by source of AA and site of digestion and absorption (Gibbs and Potter, 2002). Quality of dietary protein is important as composition of AA in dietary protein determines availability of essential AA (Gibbs and Potter, 2002). Non-essential AA, or AA which can be synthesized in the body, include alanine, asparagine, aspartate, glutamate, and serine. Dietary inclusion of these AA is less critical for survival; however, supplementation with non-essential AA may improve reproductive parameters, performance, growth, and healing (Urschel and Lawrence, 2013; Hou et al., 2015). Amino acids that can be synthesized in the body but may not be synthesized in adequate quantities under specific conditions, such as periods of growth or healing, are considered conditionally essential AA (Wu, 2009; Urschel and Lawrence, 2013). Arginine, cysteine, tyrosine, glycine, proline, and glutamine are considered conditionally essential AA (Urschel and Lawrence, 2013).

Structure of amino acids and protein

Each AA has the same basic structure: a central α-carbon, an amino group, a carboxylate group, an R group, and a hydrogen molecule (Rosenberg, 2005). A condensation reaction forms a covalent bond between the carboxylate of 1 AA and the amino group of another AA, forming a dipeptide (Rosenberg, 2005). The addition of another AA to a dipeptide forms a tripeptide. This

arrangement is continued to form a polypeptide, comprised of multiple AA. The linear sequence of AA residues is considered the primary structure of a protein (Sun et al., 2004). This primary structure will fold into repeating patterns via hydrogen bonds, forming the secondary structure. Secondary structures are primarily comprised of α-helices and β-sheets. Alpha-helices are formed through hydrogen bonding of a carboxyl group of 1 AA to the amino group of another AA within the same polypeptide chain (Eisenberg, 2003). Bonds present in α-helices are often referred to as intramolecular as they form between AA within the same polypeptide chain. Beta-sheets are formed when 2 polypeptide stands (β-strands) run parallel or antiparallel to each other and intermolecular hydrogen bonds form between the carboxyl group of 1 AA and the amino group of another AA in a parallel or antiparallel β-strand (Eisenberg, 2003). Areas where α-helices and β-sheets connect are randomly formed, increasing difficulty in determining secondary structures of protein (Sun et al., 2004). This secondary structure is further solidified into a tertiary structure by interactions - including covalent, ionic, hydrogen, and hydrophobic bonds - between R groups of amino acids (Rosenberg, 2005). The final structure of protein, or quaternary structure, results from binding multiple polypeptide chains together through covalent, ionic, hydrogen, and hydrophobic bonds (Rosenberg, 2005; Sun et al., 2004).

Solubility of dietary proteins

There are multiple methods utilized to classify proteins; however, regarding digestibility, proteins are classified as either soluble or insoluble. Proteins that are considered soluble dissolve in water or a specific solution; typically, a KOH solution is utilized to determine solubility of protein in livestock feeds (Kramer et al., 2012). Solubility of proteins is determined by binding forces within the structure. These binding forces are affected by temperature, pH, and chemical composition of solutions utilized to determine solubility (Kramer et al., 2012). Extent of

solubility is widely accepted as an estimation of digestibility of protein in monogastric animals and degradability of N by anaerobic microorganisms in ruminants (Zhao et al., 2022; Hedqvist and Udén, 2006). While solubility of protein does not always correlate with digestibility, more soluble proteins are generally associated with greater digestibility. Specific bonds present within the tertiary and quaternary structure of protein, however, may not affect solubility while impacting digestibility, and vice versa (Zhao et al., 2022).

Sources of dietary proteins

There are 3 major forms of dietary N that are included in animal feeds: animal-based proteins, plant-based proteins, and non-protein N (NPN). Animal-based proteins can be further divided into milk proteins such as casein and whey (Davidov-Pardo et al., 2015), and animal by-product proteins including fish meal, plasma, blood meal, meat meal and poultry meal (Ockerman and Basu, 2014). The most commonly used source of plant protein is soybean meal. Other sources of plant proteins include cottonseed meal, linseed meal, peanut meal, sunflower meal, cull peas (Cunha, 1990), and distiller's dried grains with solubles (DDGS; Buenavista et al., 2021). Urea is the most frequently utilized source of NPN; however, ammoniated salts and ammonia hydroxide, poultry litter, and biuret are also included in some ruminant rations (Tadele and Amha, 2015). For the purposes of this review, only sources of N that are most frequently utilized in livestock rations will be discussed.

Milk proteins are frequently utilized as sources of dietary protein due to composition and bioavailability of AA. Milk proteins are separated into 2 main categories: whey and casein (Sindayikengera and Xia, 2006). Whey is a high-quality protein source containing all essential AA (Gangurde et al., 2011), with a reported solubility between 91 and 99.7% (Mavropoulou and Kosikowski, 1973). Solubility of casein is dependent upon fraction of protein targeted and

method of treatment utilized when collecting protein from milk. One of the most common treatment methods involves mixing casein curd with sodium hydroxide, yielding sodium caseinate. Altering the structure of casein proteins through inclusion of salts yields a protein with 95 to 100% solubility when pH is between 5.5 and 7 (Zayas, 2012). However, pH ≤ 5 reduces solubility of sodium caseinate to approximately 60% (Zayas, 2012). Differences in structure between casein and whey impact site and rate of digestion. Whey has a well-formed globular structure, is associated with rapid gastric emptying, and has a reported ileal digestibility of 97 to 98% in monogastric animals (Dupont and Tomé, 2020). Casein has a more flexible structure and when pH ≤ 2, which occurs in stomachs of monogastric animals, casein elicits slower gastric emptying compared to whey (Dupont and Tomé, 2020; Dalziel et al., 2017). Casein is more sensitive to hydrolysis via gastric pepsin than whey, and it has an ileal digestibility 93 to 94% in monogastric animals (Dupont and Tomé, 2020).

Fish meal is another commonly utilized animal protein source due to its AA profile and presence of omega-3 fatty acids (Cho and Kim, 2010). Fishmeal contains approximately 37% β-sheets, 40% β-turns, 11% α-helices, and 12% random coils (Bai et al., 2016). Solubility of fish meal varies (7.2% to 33.4%) depending on source (Louw et al., 1972). Regardless of solubility, fish meal is a rumen undegradable protein (RUP), escaping microbial fermentation. Instead, it is digested in the abomasum of ruminants (Santos et al., 1998). As a result, fish meal provides ruminants with a greater variety of AA than what is provided via microbial cell protein (Santos et al., 1998) and may enhance animal performance (Broderick, 1992). Digestion of protein from fish meal by horses has not been well-evaluated; however, van Niekerk and van Niekerk (1997) reported feeding horses 200 g fish meal increased apparent total tract CP digestibility by 1.4% compared to control diets, as determined using *in vitro* techniques. It should be noted, however,

that treatments containing fish meal had an additional 200 g of protein compared to control diets (van Niekerk and van Niekerk, 1997). In horses, there are also negative palatability issues associated with feeding fish meal (Lewis, 2005).

Plasma has been included in livestock rations to increase growth parameters and is generally of porcine or bovine origin. Plasma supplementation can be in liquid, freeze-dried, or spray-dried forms, with form impacting chemical composition (Hou et al., 2019). Liquid and freeze-dried plasma both contain approximately 45% β-sheets, while spray-dried plasma is comprised of approximately 40% β-sheets. Beta-turn composition is approximately 25% in liquid and spray-dried plasma, and 33% in freeze-dried plasma (Hou et al., 2019). The greatest difference in secondary structures occurs with α-helices, whereby liquid, freeze-dried, and spray-dried plasma contain approximately 15%, 10% and 28%, respectively (Hou et al., 2019). Random coils comprised approximately 15%, 12%, and 7% in liquid, freeze-dried, and spray-dried plasma, respectively (Hou et al., 2019). As expected, solubility of protein in plasma differs based on processing, with spray-dried plasma having nitrogen solubility of 98.9% (Hou et al., 2019) and freeze-dried plasma having a nitrogen solubility of approximately 89% (Siraoukdee and Narunatsopanon, 2017). Correlating with solubility, digestibility of spray-dried plasma protein by pepsin and trypsin *in vitro* is 97.3%, whereas digestibility of freeze-dried plasma protein is 93% (Uchman and Konieczny, 1989). Preileal digestibility of spray-dried plasma protein in pigs is 81.8% to 85.3% (Jeong et al., 2016; Almeida et al., 2013) while preileal digestibility of freeze-dried and liquid plasma protein is not well recorded.

Soybean meal is one of the most utilized sources of N in livestock feeds due to its relatively high protein content and low cost (Zhang et al., 2017). There are, however, several anti-nutritional factors that impact the digestibility of protein in raw soybean meal. Anti-

nutritional factors in soybean meal include lectins (glycoproteins that interact with sugar molecules and are indigestible by mammalian enzymes), phytic acids (acids which interact with minerals and reduce digestibility by mammalian enzymes), and protease inhibitors (Yasothai, 2016). Fortunately, thermal processing reduces the presence of anti-nutritional factors, thus increasing digestibility (Adeyemo and Onilude 2013; Banaszkiewicz, 2011). The secondary structure of soybean meal is approximately 41% β-sheets, 40% β-turns and 19% α-helices, while random coils were not detected (Yasar et al., 2020). Others have reported slightly different proportions, with approximately 33% β-sheets, 36% β-turns, and 12% α-helices, with 12% random coils (Bai et al., 2016). Generally, the reported KOH solubility of protein in soybean meal is approximately 80% (Karr-Lilienthal et al., 2004). Farley et al. (1995) fed increasing levels of dietary N supplied via soybean meal and reported true post-ileal digestibility of nitrogen to be 89.8% and total tract digestibility of 95.7%.

Urea is a source of NPN that may be included in ruminant rations as a source of affordable N. Within the rumen, microorganisms degrade urea into ammonia, which is then transformed into amino acids by microorganisms and utilized for cellular maintenance and proliferation. In this manner, N requirements of microorganisms are met while microbial cell protein (MCP) provides the animal with required N and a variety of AA (Tadele and Amha, 2015). However, inclusion of NPN in equine rations, while not necessarily harmful, does not benefit the horse due to prececal digestion of nitrogen (Martin et al., 1995).

Digestion of protein in the horse

Foregut digestion

Digestion of protein in the equine foregut occurs in the same manner as in monogastric animals. Hydrochloric acid secreted by gastrin cells in the equine stomach denature quaternary

and tertiary structures of protein (Urschel and Lawrence, 2013). Hydrochloric acid also serves to reduce gastric pH, subsequently activating the zymogen pepsinogen to pepsin. Pepsin acts as an endopeptidase, randomly cleaving between AA within a polypeptide chain, yielding 2 smaller peptide chains (Urschel and Lawrence, 2013). In addition to gastric enzymes, proteolytic microorganisms are found within the equine stomach. Perkins et al. (2012) reported the most abundant phylum in the gastric region of the equine stomach is Proteobacteria. The genera *Moraxella* (Yoshimoto et al., 1995) and *Acinetobacterium* (Muhammed et al., 2021) are both within the Proteobacteria phylum and have protease activity. Species of *Prevotella* (*P. ruminocola*, *P. brevis*, *P. albensis*, and *P. bryantii*) also have proteolytic activity (Griswold et al., 1999). *Prevotella* has been isolated in the equine stomach, though specific species of *Prevotella* in the horse have yet to be identified (Perkins et al., 2012). The extent to which proteins are degraded by microbial enzymes in the equine stomach is dependent upon composition of the microbial population and dietary source of N (Griswold et al., 1999). Despite presence of mammalian and microbial proteolytic enzymes, there is limited information regarding extent of degradation and absorption of protein in the equine stomach. Digesta, however, is only in the equine stomach for approximately 2 to 6 h (Van Weyenberg et al., 2006). As a result, gastric digestion of nutrients is limited by rapid ROP.

Activity of proteolytic enzymes is greatest in the small intestine compared to other sections of the equine gastrointestinal tract (Hintz, 1975). Thus, the small intestine is the main site of digestion of protein in equines. Enterokinase, a proteolytic enzyme located on the brush border of the small intestine, activates other proteolytic enzymes in the small intestine including trypsin, chymotrypsin and elastase (Lainé et al., 1993). Activation of trypsin also serves to activate chymotrypsin and elastase, as well as procarboxypeptidases (Merritt and Juilland, 2013).

Trypsin is classified as an endopeptidase and cleaves peptide bonds following arginine or lysine residues, yielding smaller oligopeptide chains (Baird et al., 2013). Procarboxypeptidases cleave individual AA from the carboxyl end of peptide chains (Merritt and Juilland, 2013). Chymotrypsin separates long-chain and short-chain oligopeptides by cleaving peptide bonds following the carboxyl groups of tryptophan, phenylalanine, tyrosine and leucine (Gráf et al., 2013). Peptides containing a neutral charge are hydrolyzed by oligopeptidases located on the brush border of the small intestinal lumen, yielding single AA, di-, and tripeptides (Merritt and Juilland, 2013).

Proteins and peptides are also degraded by microorganisms present throughout the small intestine including strains of *Streptococcus* and *Lactobacillus* (Rodríguez-Serrano et al., 2018). Protease activity is specific to individual microorganisms and may be AA-specific, but that discussion is beyond the scope of this review. Regardless of enzymatic source, the NRC (2007) estimates approximately 51% of dietary protein is digested pre-cecally; however, Hintz (1975) estimated 70% of protein may be digested prior to the cecum. The percentage of N digested pre-cecally depends on form of dietary protein. Proteins present in roughages are generally less accessible than proteins in cereal grains due to peptides being bound to cellulose and hemicellulose (Zeyner et al., 2015).

After dietary proteins are broken down, individual AA and small peptides are absorbed through the small intestinal lining. Within the equine small and large intestine, Woodward et al. (2014) reported several solute carrier (SLC) family genes associated with AA acid transport. The genes of interest code for $b^{0,+}$AT, CAT-1, LAT-2, and LAT-3 AA transporters, all of which are sodium-independent systems. Expression of SLC7A9, SLC7A1, SLC7A8, and SLC43A1 did not differ between the jejunum and ileum (Woodward et al., 2014). Authors, however, did not

evaluate gene expression of SLC family transporters in the equine duodenum. Absorption of N in the small intestine is approximately 11 to 30% of total tract digestibility of N (Mok and Urschel, 2020). A goal of protein digestion and absorption in the horse is to increase N absorption in the foregut. This is due to greater variety of AA yielded during digestion of protein in the small intestine compared to the hindgut. It is important adequate N reaches the hindgut to meet microbial requirements. There is, however, little research available regarding digestibility of protein in the equine hindgut. Any nitrogenous compounds that escape small intestinal digestion and absorption will enter the cecum, the first section of the equine large intestine, and undergo microbial fermentation.

Hindgut fermentation

The equine hindgut contains a dense population of anaerobic microorganisms that ferment a variety of nutrients, including carbohydrates and proteins. Due to limited availability of information regarding the equine cecum, however, the rumen often serves as a model for microbial fermentation in the equine hindgut. While all microorganisms require AA for cellular maintenance and proliferation, only specific microorganisms produce the necessary enzymes to break down peptides. Non-proteolytic microorganisms rely on proteolytic microorganisms to degrade peptide bonds to yield individual AA, or they utilize non-protein sources of nitrogen such as ammonia produced during the fermentation process. Fermentation of protein by proteolytic ruminal microorganisms is bi-phasic. Initially, dipeptidyl aminopeptidases cleave dipeptides from larger peptides chains (Wallace, 1996). Once dipeptides are liberated, the second phase of degradation of peptides occurs when dipeptidases cleave individual amino acids from liberated dipeptides (Wallace, 1996).

The rate and extent in which protein is fermented by microorganisms depends on source and structure of protein, as well as populations of microorganisms within a community (Griswold et al., 1999). Nitrogen supplied via casein is rapidly degraded, with a half-life between 5.6 and 21.5 min when casein was dosed directly into the rumen of cattle (Mangan, 1972). Rate of passage, however, is not factored into half-life of fermentation of protein. When comparing rate of degradation of soluble protein *in vitro*, Hedqvist and Úden (2006) utilized casein as a control, with a fractional rate of degradation (fraction of protein degraded$\cdot h^{-1}$) of 1.00 and an effective protein degradation [fractional rate of degradation/(fractional rate of degradation + fractional rate constant of passage)] of 0.85. Fractional rate of degradation and effective protein degradation of wheat DDGS (0.62 and 0.79, respectively) and soybean meal (0.46 and 0.73, respectively) were statistically similar, but were less than casein (Hedqvist and Úden, 2006). Broderick et al. (2004) reported an average rate of degradation of casein protein to be 0.286 and an average ruminal escape of 17.2%. Meanwhile, soybean meal had a reported rate of degradation of protein of 0.152 and 29% ruminal escape protein (Broderick et al., 2004). Differences reported in degradation of solubility of proteins between Hedqvist and Úden (2006) and Boderick et al. (2004) is due to application of inhibitors preventing measurement of peptides and the use of linear models. The use of non-linear models, as utilized by Hedqvist and Úden (2006), more closely mimics *in vivo* degradation of proteins. Amid the protein sources utilized in livestock feeds, casein is not only highly soluble, but it does not contain disulfide bridges, allowing more rapid fermentation by microorganisms (Hedqvist and Úden, 2006).

The Cornell Net Carbohydrate and Protein System classifies nitrogen into 3 main categories, rapidly degradable NPN (fraction A), conditionally degradable true protein (fraction B), and not available true protein (fraction C); fraction B is further separated into soluble in

borate phosphate buffer (B1), insoluble in neutral detergent (B3), and the difference between B1 and B3 fragments (B2; Lanzas et al., 2007). Extent to which fraction B is degraded is partially dependent on rate of passage as well as the composition of B fraction proteins. Protein present in soybean meal is 13.5% fraction A, 85.9% fraction B, and 0.5% fraction C (Romagnolo et al., 1990). Fraction B for soybean meal is further separated in 21.5% B1, 21.3% fraction B2, and 43.1% fraction B3 (Romagnolo et al., 1990). Soybean meal contains 15.7 to 31.8% albumins, 30.2 to 46.5% globulins, 16.1 to 38.5% glutelins, and 0.20 to 0.82% prolamins (Ciabotti et al., 2016). Secondary structures present within globulins of soybean meal, however, are connected through disulfide bonds, which are resistant to microbial fermentation (Singh et al., 2015). Additionally, glutelins (a protein fraction within promalins) contain several disulfide bonds, increasing structural stability and decreasing efficacy of microbial proteases to degrade glutelins (Romagnolo et al., 1994). As a result, soybean meal is not as rapidly fermented by microorganisms as casein. Wheat DDGS contains predominantly glutelins (40%), followed by approximately 31% gliadins, 19% albumins, and 10 % globulins (Chatzifragkou et al., 2016). Differences in protein structure (albumins, globulins, glutelins, promalines) impacts the extent by which microorganisms are able to degrade proteins, with greater quantities of albumins and globins increasing rate and extent of fermentation compared to promalins.

Another method to evaluate degradation of protein is through enumeration of proteolytic microorganisms; as protein availability increases, abundance of proteolytic microorganisms should increase. Ruminal populations of proteolytic microorganisms in dairy cows differed when cultured utilizing soybean meal (11.0×10^6) compared to *Leucaena leucocephala* (9.2×10^6) and yeast-fermented cassava chips (YEFECAP: 13.3×10^6) as substrate, while cassava hay yielded similar populations (12.1×10^6) compared to soybean meal and yeast-fermented cassava chips

(Wanapat et al., 2011). Source of protein, however, had no effect on total populations of microorganisms (Wanapat et al., 2011). *Leucaena leucocephala* is a legume with leaves containing approximately 23% CP. *Leucaena leucocephala* leaves, however, also contain 2.7% tannins and 3.1% mimosine, both of which are toxic to ruminal microorganisms (Wanapat et al., 2011). As a result, the antinutritional factors in leaves of *Leucaena leucocephala* likely limited growth of proteolytic microorganisms in cultures containing this source of protein. This serves as an example of why antinutritional factors must be taken into consideration when evaluating protein digestibility of a feedstuff. When studying microbial populations throughout the equine gastrointestinal tract, Mackie and Wilkins (1988) reported 16.5×10^8 equine cecal microorganisms grew on media containing casein as the primary substrate. Meanwhile, 19.4×10^8 ruminal microorganisms grew on casein media (Mackie and Williams, 1988). Proteolytic microorganisms are likely more prolific in ruminal contents compared to cecal contents due to prececal digestion of protein in the horse.

The genera *Prevotella* and *Bacteroides*, both within the Bacteroidota phylum, have been identified as primary proteolytic microorganisms in the bovine rumen (Flint and Duncan, 2014). Historically, the primary specie within the *Prevotella* genus was *P. ruminicola*, but this specie has been reclassified to consist of 4 different species: *P. ruminocola, P. brevis, P. albensis, P. bryantii* (Flint and Duncan, 2014). Species within *Prevotella* produce both dipeptidyl aminopeptidases and dipeptidases, fermenting protein in both the primary and secondary stage of degradation in the bovine rumen (Wallace, 1996). Enzymes produced by *P. ruminicola* and *P. brevis* include cysteinases and metallopeptidases (Griswold et al., 1999). *Prevotella albenis, P. bryantti*, and *P. sp2202* produce serinases and metalloproteases (Griswold et al., 1999). While specific species of *Prevotella* have yet to be identified in the equine hindgut, the family

Prevotellaceae and the genus *Prevotella* have been documented (Collinet et al., 2021; Kauter et al., 2019; Sorenson et al., 2021). Sorensen et al. (2021) reported greater average relative abundance of *Prevotella* in cecal contents when horses were offered alfalfa vs bromegrass hay, as well as in cecal contents compared to feces. Authors attributed these differences to greater protein and sCHO in alfalfa compared to bromegrass and in the cecum compared to rectum (Sorensen et al., 2021).

Bacteroides have also been identified in the equine cecum (Warzecha et al., 2017; Daly et al., 2001), large colon (Daly et al., 2001) and feces (Tavenner et al., 2020). Specific proteolytic activity of *Bacteroides* in the horse is currently unknown; however, species within this genus have been proven to degrade protein in ruminants. Of the enzymes produced within different strains of the species *Bacteroides amylophilus* approximately 40% are proteases, with many proteases being membrane bound (Blackburn, 1968). Species within the *Bacteroides* genera produce serine proteases (Flint and Duncan, 2014; Jessop et al., 1993; Blackburn, 1968) and degrade a variety of sources of N, including proteins in casein, trypsin, chymotrypsin, azocasein and azosoya bean flour (Gibson and MacFarlane, 1988). Proteins found in components of blood, however, are poorly degraded by *Bacteroides* (Gibson and MacFalane, 1988). While not primary fermenters of protein, other microorganisms that produce proteolytic enzymes include the genera *Streptococcus, Butyrivbrio* (Sales-Duval et al., 2014; Mackie and White, 1990), *Lachnospira*, *Eubacterium, Clostridium, Propionibacterium, Succinivbrio*, *Selenomonas*, and *Megasphaera* (Mackie and White, 1990).

Microbial fermentation of proteins yields short chains of polypeptides, single AA, and ammonia. Amino acids are transported across the intestinal lining via transporters. Woodward et al. (2014) reported mRNA coding for 4 AA transporters throughout the gastrointestinal tract of

the horse. Messenger RNA for all transporters were detected in the cecum, large colon and small colon; however, the concentration of mRNA for the transporters differs upon segment (Woodward et al., 2014). The presence of mRNA for AA transporters suggests AA are absorbed in the hindgut of the horse. When feeding horses a diet containing casein as the primary source of N, Nelson and Tyznik (1971) measured greater plasma concentration of ammonia in the cecal portal vein compared to the jugular vein. This implies ammonia was absorbed through the cecal lining of the horse. L-lysine absorption has been documented in the equine hindgut, with greater absorption in the proximal colon compared to the distal colon (Woodward et al., 2012). In contrast, Bochröder et al. (1994) reported no *in vitro* transport of lysine, histidine, or arginine across the mucosa of ventral colon collected from ponies when concentrations of these AA were within physiological range. *In vitro* absorption of ammonia across mucosa of ventral colon was approximately 63%, demonstrating a greater capacity for absorption of ammonia compared to AA in the equine hindgut (Bochröder et al., 1994). It should be noted, however, there is limited information regarding absorption of AA and ammonia in the equine hindgut due to difficulty associated with sampling.

The concentration of nitrogenous compounds available for absorption across the intestinal lining also depends on N utilization by microorganisms present in the equine hindgut. All microorganisms, not just proteolytic microorganisms, utilize single AA, short-chain polypeptides, and ammonia as sources of nitrogen for maintenance and proliferation. The ability to transport nitrogenous compounds through the membrane of microorganisms is species-specific; however, species within the same genera will typically share common transporters. Some transporters will preferentially transport short-chain polypeptides, saving energy as the cost to transport single AA is the same as transporting 4 amino acids bound together (Kieliszek

et al., 2021). The concentration of AA and ammonia present in the system (cecum, large colon, or small colon), however, will impact specificity and rate of transport of nitrogenous compounds into microorganisms (Kieliszek et al., 2021). Nitrogen is required for proliferation and repair by hindgut microorganisms, with certain microorganisms requiring specific AA or branch-chain (BCAA) present in sodium caseinate (Griswold et al., 1996; Köster et al., 1996). Due to prececal digestion, however, protein – and specific AA yielded from fermentation of protein – is likely a limiting factor for microbial growth. Subsequently, fermentation of fiber in horses consuming poor-quality forage may be limited (Santos et al., 2013). Protein bound to hemicellulose and cellulose is indigestible in the small intestine, thus it escapes the foregut and thereby provides limited quantities of N to equine hindgut microorganisms upon its liberation from these fibrous substances during microbial fermentation. Approximately 7% rumen degradable protein meets microbial requirements for nitrogen in the rumen (Bergman, 1990). Microorganisms in the equine hindgut likely require a similar amount of protein, as dosing casein directly into the cecum of horses had no effect on fermentation parameters when horses were fed forage containing 8.5% CP (Jordan et al., 2018). Quantity of protein entering the cecum, however, was not measured. In contrast, Oliveira et al. (2015) recommends feeding 11.4% dietary protein to maximize digestibility of neutral detergent fiber in exercising horses. A major limitation of both studies was lack of data regarding presence of protein in the equine hindgut specifically. Any nitrogenous compound not utilized by microorganisms or absorbed in the equine hindgut is wasted and expelled in the feces.

Presently, microbial requirement of N is unknown; however, in order to maximize microbial growth and efficiency, N and energy must be available simultaneously so neither protein nor energy is wasted (Hackmann and Firkins, 2015; Santos et al., 2013). Therefore, it is

necessary to estimate proportion of dietary protein which may escape digestion in the equine foregut and to supply carbohydrates that will be fermented by microorganisms at approximately the same rate as protein entering the hindgut.

Carbohydrate digestion in the horse

Introduction

Carbohydrates (CHO) are an immensely important component of the equine diet, serving as the primary energy source for horses. Carbohydrates can be classified on a variety of criteria; however, in this review CHO are separated into 2 groups: non-structural CHO (nsCHO) and structural CHO (sCHO). Non-structural CHO are the energy storage components of plants and include simple sugars, short fructooligosaccharides, amylodextrins, and starch, most of which can be digested and absorbed in the small intestine of the horse (Chesson and Forsberg, 1997). Structural CHO, commonly referred to as fiber, are components of cell walls of plants and include lignin, hemicellulose, and cellulose (Chesson and Forsberg, 1997). While sCHO comprise a major fraction of the equine diet, horses lack the necessary enzymes to digest sCHO. An exception is pectin, which is also classified as a sCHO. Amylopectinase, which is secreted in the small intestine, allows for restricted degradation of pectin (Hoffman, 2009). To obtain energy from these fibrous components of feed, microorganisms present throughout the hindgut of the horse ferment sCHO, yielding VFA. These VFA are utilized by microorganisms for growth and proliferation, or they can be absorbed through the intestinal lining and used as an energy source for the horse. While lignin contributes to the rigidity of plants, it is completely indigestible by enzymes of either equine or microbial origin (McSweeney et al., 1994; Raffrenato et al., 2017), and thus lignin will not be further discussed in this review.

Digestion of CHO in the equine foregut

Digestion of nutrients is initiated during mastication, whereby feed is ground into smaller particles, increasing surface area. Increased surface area allows greater access for mammalian and microbial enzymes to degrade nutrients. As a result, the degree to which feed is masticated can impact digestion. Equine saliva contains limited α-amylase activity and therefore does not significantly contribute to digestion of starch. Instead, equine saliva plays a more important role in buffering gastric contents, increasing gastric pH, and aiding in the control of gastric ulcers (McSweeney et al., 1994; Raffrenato et al., 2017). Saliva secretion in the horse is a tactile response, meaning the act of chewing stimulates saliva secretion. Therefore, horses produce greater quantities of saliva when consuming their natural, fiber-dense diet – which requires more chewing - compared to grain-rich diets often fed to performance horses (Meyer et al., 1985).

The equine stomach does not secrete enzymes required to degrade CHO; however, low gastric pH results in acidic hydrolyzation of nsCHO. The equine stomach has a pH gradient, with the non-glandular portion having a pH of approximately 4 and the glandular region having a pH of 2 to 2.5 (Al Jassim, 2006). Degradation of nutrients may be dependent on this stratification within the equine stomach. Supporting this, hydrolyzation of fructans *in vitro* was greater at pH 4 compared to pH 2 and 3 (Strauch et al., 2017). Strauch et al. (2017) suggested enzymes within plant matter, which are more active at pH 5, may have been responsible for the increased disappearance of DM in cultures with pH 4 compared to pH 2. Microorganisms attached to gastric mucosa also aid in degradation of nsCHO. Among the microorganisms isolated from the equine stomach, species of *Lactobacilli* and *Streptococcus* rapidly ferment simple sugars and starch (de Fombelle et al., 2003; Costa et al., 2015). These species are more acid tolerant than other species and are found in greater abundance in the glandular region compared to the non-

glandular or antral regions of the stomach (Perkins et al., 2012). Lactic acid, a product of fermentation of starch by *Lactobacillus* and *Streptococcus*, then serves as substrate for *Veillonella*, which yields the VFAs propionate and acetate from utilization of lactate (Ng and Hamilton, 1971; de Fombelle et al., 2003; Biddle et al., 2013; Costa et al., 2015). Consequently, increased gastric populations of *Lactobacillus* (8.4 $\log_{10}$ vs. 7.8 $\log_{10}$) and *Veillonella* (7.6 $\log_{10}$ vs. 6.8 $\log_{10}$) are present when feeding a nsCHO-rich diet to performance horses compared to a maintenance diet (de Fombelle et al., 2003). Despite these digestive mechanisms, digestion of nsCHO in the equine stomach is limited by a rapid ROP (Van Weyenberg et al., 2006).

Most nsCHO are digested in the equine small intestine where pancreatic α-amylase cleaves α-1,4 glycosidic linkages within polysaccharide chains (Dyer et al., 2002; Hoffman, 2009). While pancreatic amylase is the primary enzyme for degradation of polysaccharide chains comprising nsCHO in the equine small intestine, activity of pancreatic amylase is limited compared to monogastric species (Alexander and Hickson, 1970). Lorenzo-Figueras et al. (2007) reported a concentration of 2.3 U amylase/mg pancreatic protein in the horse, while pigs and rats had 107 and 56 U amylase/mg pancreatic protein, respectively. Meanwhile, calves also had 2.3 U amylase/mg pancreatic protein (Lorenzo-Figueras et al., 2007). After nsCHO have been degraded into short-chain oligosaccharides and di- and trisaccharides, sugar-specific enzymes (lactase, maltase, and sucrase) located on the brush border of the luminal surface yield individual sugar molecules (Dyer et al., 2002). Lactases hydrolyze the β-1,4 bond between glucose and galactose molecules (Stylianopoulos, 2005). Maltose is separated into 2 glucose molecules through hydrolyzation of the α-1,4 bond by maltases, and sucrases hydrolyze the α-1,4 bond between glucose and fructose molecules in sucrose. Activity of lactase and sucrase is greater in

the duodenum compared to the ileum (Dyer et al., 2002; Alrammahi, 2020). Activity of maltase, however, is similar throughout the equine small intestine (Dyer et al., 2002; Alrammahi, 2020).

Hemicellulose and cellulose, both sCHO, are not degraded via mammalian enzymes; thus, these fibrous components are not broken down in the equine small intestine. While functionally classified as a sCHO, pectin may be partially degraded in the equine small intestine by amylopectinase, which hydrolyzes α-1,6 glycosidic bonds that attach side chains to the main glycosidic chain of pectin (Hoffman, 2009).

In addition to pancreatic and brush border enzymes, the equine small intestine also hosts anaerobic microorganisms. De Fombelle et al. (2003) cultured amylolytic (*Streptococci* and *Lactobacilli* genera), cellulolytic, and lactic acid-utilizing microorganisms, and they reported populations of these microorganisms did not differ between the jejunum and ileum (de Fombelle et al., 2003). Ericcson et al. (2016) confirmed *Lactobacillus* sp. and *Streptococcus* sp. are present in the jejunum and ileum of horses, though average relative abundance varied greatly between horses. While the presence of these microorganisms indicates a microbial contribution to small intestinal digestion and absorption of nsCHO, this contribution is likely minor due to a rapid ROP through the small intestine. While fibrolytic microorganisms have been detected in the equine small intestine, the average relative abundance of the major fibrolytic microorganisms *F. succinogenes* and *R. flavefaciens* is 0.01% to 0.02% (Ericcson et al., 2016). Due to restricted average relative abundance and rapid ROP, there is no evidence of fermentation of fiber in the small intestine (Coenen et al., 2006).

Simple sugars yielded through enzymatic digestion are absorbed across the intestinal lumen. Glucose and galactose are transported via the Na^+/glucose cotransporter (SGLT1 protein; Roberts; 1975; Dyer et al., 2002). Absorption of glucose decreases as digesta moves through the

small intestine, with the greatest abundance of SGLT1 present in the duodenum and jejunum (Dyer et al., 2002). Fructose is absorbed via the GLUT5 transporter (Hoffman, 2013). Similar to SGLT1, the greatest concentration of GLUT5 transporters is in the duodenum, followed by the jejunum, and lastly the ileum (Hoffman, 2013).

The capacity of the small intestine to digest and absorb nsCHO varies greatly, partly due to dietary adaption and differences in source and processing of CHO (Kienzle et al., 1997). Dyer et al. (2009) reported that gradual acclimation of horses to diets containing 2 g starch·kg BW^{-1}·$meal^{-1}$ resulted in increased expression of SGLT1 protein in the jejunum and ileum, allowing for greater absorptive capacity of simple sugars. It should be noted that botanical origin of nsCHO drastically alters pre-cecal digestibility, primarily due to variations in structures of bonds tethering the various monosaccharides together. Keinzle et al. (1997) demonstrated the impact of botanical origin on pre-cecal digestibility, with whole corn, barley, and oats having 29, 22, and 80% pre-ileal digestibility, respectively. Pin holes, or entry points for enzymes to access starch molecules, within each grain type vary, with oats having more microscopic pin holes than barley or corn (Keinzle et al., 1997). Grinding corn increases pre-ileal digestibility to somewhere between 47 to 71% (Kienzle et al., 1997). Similarly, grinding oats and barley increases pre-ileal digestibility compared to rolled or whole grains (Julliand et al., 2006). Pre-cecal digestibility of starch is maximized with heat treatment of grains, which leads to gelatinization of starch molecules (Julliand et al., 2006; Vervuert et al., 2003).

Digestibility of starch is also dependent upon amount of offered. Pre-cecal digestibility of starch is approximately 80 to 96% of total starch intake when feeding horses 1.2 to 1.5 g starch·kg BW^{-1}·$meal^{-1}$ (Potter et al., 1992). Increasing dietary starch to 2.2 g starch·kg BW^{-1}·$meal^{-1}$, however, reduced prececal digestibility to 59% of total starch intake, with maximum

starch willingly consumed being 3.5 g starch·kg BW^{-1}·$meal^{-1}$ (Potter et al., 1992). Based off this research, the current recommendation for maximum inclusion of dietary starch in equine rations is 2 to 4 g starch·kg BW^{-1}·$meal^{-1}$.

Digestion of CHO in the equine hindgut

Horses evolved to extract energy from poor-quality roughages through a symbiotic relationship with microorganisms residing in the hindgut. These microorganisms secrete specialized enzymes that degrade fibrous components of feed, specifically cellulose, hemicellulose, and pectin. They may also degrade starch, simple sugars, and proteins, and liberate fatty acids from alcohol backbones of lipids. Microorganism are classified according to their preferred substrate, with the primary functional groups being fiber-degrading (fibrolytic), which can be separated into cellulose (cellulolytic) and hemicellulose-degrading (xylantic), starch and sugar-degrading (amylolytic), and lactate-degrading (lactate-utilizing; Collinet et al., 2021; Kauter et al., 2019). Regardless of substrate, end products of microbial fermentation are volatile fatty acids (VFA). These VFA are utilized by microorganisms at the site of production in the lumen of the digestive tract, by colonocytes, or by the animal following absorption across the intestinal lining (Bergman, 1990). The most common VFA produced from microbial fermentation include acetate, propionate, and butyrate, with intermediate fatty acids including lactate and succinate (Bergman, 1990). Volatile fatty acids are absorbed across the intestinal lining and made available to the horse via the transport protein monocarboxylate transporter (MCT-1; Nedjadi et al., 2014). This protein exchanges 1 ionized VFA molecule for 1 molecule of H_3O^{-1}, and will preferentially transport acetate, propionate, and lactate over butyrate (Nedjadi et al., 2014). The proportion by which these VFA are produced vary according to diet and impacts how much energy is available to the horse (Bergman, 1990). Typically, feeding diets

rich in fiber results in greater proportions of acetate, whereas diets rich in nsCHO promote the production of propionate and butyrate (Bergman, 1990). Propionate is the most gluconeogenic VFA and can be directly transformed into glucose by the horse (Bergman, 1990). Butyrate is utilized by colonocytes to maintain integrity of the intestinal lining (Nedjadi et al., 2014). Excess butyrate, however, may be converted to acetate, or it may be absorbed and utilized as an energy source for the horse (Bergman, 1990). Acetate is a major VFA produced during cecal fermentation and, once absorbed, plasma acetate can be oxidized, serving as source of energy in tissues, or contribute to synthesis of fatty acids and adipose tissue (Shepard et al., 2014).

Fermentation of structural CHO

When consuming an all-forage diet, the microbial population within the cecum of the horse is primarily composed of fibrolytic bacteria that degrade cellulose and hemicellulose. The 2 most dominant fibrolytic microorganisms found in the equine cecum are within the *Fibrobacter* and *Ruminococcus* genera (Julliand et al., 1999; Julliand and Grimm, 2017). To date, 2 species within the *Fibrobacter* genus have been isolated from the horse, *F. succinogenes* and *F. intestinalis* (Neumann et al., 2017), with *F. succinogenes* being reported most frequently. Degradation of cellulose by *F. succinogenes* is dependent upon cellulose binding molecules, which attach the microorganism to the cellulose molecule (Neumann et al., 2018). The primary enzymes produced by *F. succinogenes* includes a variety of cellulases and xylanases, which are often referred to as hemicellulases (Neumann et al., 2018). *Fibrobacter succinogenes* can also degrade glucose via endoglucanases (Neumann et al., 2018), with glucose being more rapidly fermented, though populations of *F. succinogenes* are not maintained when glucose is the only available source of energy (Raut et al., 2015). The genus *Ruminococcus* comprises approximately 5.85% of the average relative abundance of microorganisms in the equine cecum

(Hastie et al., 2008) and contains both amylolytic (*R. callidus)* and fibrolytic species *(R. albus, R. flavefaciens; Daly et al., 2001).* Of the species within the *Ruminococcus* genus, *R. flavefaciens* is considered the greatest contributor to degradation of fiber and can utilize cellulose, xlyans, galacturonic acid, raffinose, maltose, lactose, sucrose, galactose, glucose, and xylose as energy substrates (Juilland et al., 1999). Similar to *F. succinogenes*, *R. flavecians* and *R. albus* require cellulose binding proteins to adhere to the surface of cellulose molecules and produce a variety of cellulases, xylanases, and pectinases (Privé et al., 2015).

Other microorganisms that contribute to fiber degradation within the equine hindgut include *Treponema*, *Prevotella*, and *Lachnospiraceae*. While individual species of *Treponema* in the equine hindgut have not been identified to date, Daly et al. (2001) reported 6 sequences clustered closely with *T. byrantii* and *T. succinifaciens*, and it seems likely the equine counterparts fill a role similar to these ruminal microorganisms. *Treponema byrantii* utilizes products liberated from cellulose degradation, including glucose and cellobiose, and, therefore, relies on activity of other fibrolytic microorganisms (Stanton and Canale-Parola, 1980). In cultures prepared utilizing ruminal inoculum, the presence of *T. byrantii* enhanced fermentation of cellulose by *F. succinogenes*, demonstrating a symbiotic relationship between these microorganisms (Stanton and Canale-Parola, 1980). *Treponema succinifaciens* is a saccharolytic microorganism that utilizes pentoses, hexoses, and disaccharides, yielding large quantities of succinate (Edwards et al., 2020). Succinate is metabolized into propionate (Bergman, 1990) via the succinic pathway (Nozière et al., 2010). Several microorganisms, including the genera *Veillonella*, *Phascolarctobacterium*, *Selenomonas*, *Clostridium*, and *Megasphera*, are capable of producing the enzymes necessary to utilize succinate as an energy source, yielding propionate (Reichardt et al., 2014). Specific species of the *Prevotella* genus have not been identified in the

hindgut of the horse. Species of *Prevotella* isolated from ruminants, however, degrade a variety of substrates including hemicellulose (*P. albensis*, *P. brevis*, *P. bryanti*, and *P. rumincola*), cellobiose (*P. rumincola*), various simple sugars (*P. rumincola*), and protein (*P. ruminicola*; Lou et al., 1996; Flint and Stewart, 1999; Nagaraja, 2016). Therefore, *Prevotella* is regarded as both fibrolyitc and saccharolytic, depending on specie or strain. Excess glucose, however, is toxic to some species of *Prevotella* (Russell, 1993). Within the family *Lachnospiraceae*, there are a variety of genera including *Butyrivbrio, Blautia, Coprococcus, Dorea, Lachnospira, Oribacterium,* and *Roseburia* (Vacca et al., 2020). *Butyrivibrio* has been identified in bovine ruminal contents and has been associated with the degradation of hemicellulose (Cotta and Forster, 2006) and pectin (Gradel and Dehority, 1972). The presence of other substrates, such as sucrose, however, down-regulates activity of enzymes produced by *B. fibrisolvens* necessary to ferment xylans present within hemicellulose (Russell and Baldwin, 1978). *Butyrivibrio fibrisolvens* has also been associated with protein degradation and attachment to cellulose (Cotta and Hespell, 1986).

Fermentation of non-structural CHO

There are a variety of microorganisms that degrade simple sugars, including the aforementioned fibrolytic bacteria and genera within the *Selenomondaceae*, *Streptococcaceae*, and *Lactobacillaceae* families. *Selenomonas ruminatum*, a ruminal microorganism, degrades lactose, maltose, and glucose, with specific subspecies fermenting lactate and glycerol. Propionate and succinate are the primary VFA produced by *S. ruminantum*, with acetate being produced in lesser quantities (Nagaraja and Titegemeyer, 2007). The primary saccharolytic microorganisms responsible for the degradation of starch in the equine cecum, however, are *Streptococcus* and *Lactobacillus*. *Streptococcus bovis* and *S. equinus* have been isolated from the

gastrointestinal tract of healthy horses (Al Jassim and Rowe, 1999; Harlow et al., 2016). It has been suggested that *S. bovis* and *S. equinus* are synonymous (Gobbetti and Calasso, 2014); however, Al Jassim and Rowe (1999) reported the presence of both microorganisms in equine fecal matter, differentiating the species by substrates utilized. *Streptococcus equinus* strains are saccharolytic but do not ferment starch or inulin (Al Jassim and Rowe, 1999). All strains belonging to *S. bovis*, however, utilized cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, starch, and sucrose as substrate (Al Jassim and Rowe, 1999). Despite differences in substrate utilization, *S. bovis* and *S. equinus* are typically reported as either the same microorganisms or as a *S. bovis* – *S. equinus* complex, as both are saccharolytic and produce lactic acid as a product of fermentation (Al Jassim and Rowe, 1999; Gobbetti and Calasso, 2014; Kauter et al., 2019). *Lactobacillus bifidus*, *L. salivarius,* and *L. mucosae* also have been detected in the equine gastrointestinal tract (Al Jassim, 2005; Juilland and Grimm, 2016). Species within the *Lactobacillus* genus rapidly degrade starch, amylodextrins, maltose, and glucose, yielding lactic acid (Nagaraja and Titegemeyer, 2007).

In forage-fed horses, relative abundances of *Streptococcus* and *Lactobacillus* in the equine cecum are 0.32% to 0.37% and 1.26% to 3.08%, respectively, depending on the type of forage consumed (Sorensen et al., 2019). When feeding large quantities of nsCHO, as is common in performance horses, relative abundances of these genera increase. Daly et al. (2012) compared the cecal microbiome of horses consuming a forage-only diet to that of horses consuming a concentrate and forage diet. Horses consuming concentrate also had greater relative abundance of the Bacteroidetes assemblage (24.7% vs. 13.5%) and the *Bacillus* – *Lactobacillus* – *Streptococcus* group (5.5% vs. 3.9%) compared to horses only consuming forage (Daly et al., 2012). Consequently, as the amylolytic *Bacillus* – *Lactobacillus* – *Streptococcus* group

increased, there was a subsequent reduction in average relative abundance of *Fibrobacter* (2.7 % vs. 0.5%) and *Ruminococcaceae* (1.4% vs. 0.3%) in horses consuming concentrate compared to horses consuming a forage-only diet (Daly et al., 2012).

In healthy horses that are well adjusted to diets rich in nsCHO, nsCHO entering the cecum provide substrate to a variety of microorganisms. When feeding horses a diet containing concentrates, molar concentration of lactate was greater (2.5) compared to horses consuming an all-forage diet (1.0); however, concentrations of acetate, propionate, butyrate, valerate, and isovalerate were consistent (Daly et al., 2002). The removal of excess VFA - and fermentation of intermediary products (lactate) from degradation of starch - are imperative for maintaining a healthy microbiome. An important functional group of microorganisms necessary to clear these intermediary products are lactate-utilizing bacteria. Known lactate-utilizing microorganisms in the equine hindgut include *Veillonella* sp., *Megasphera elsdenii*, and *Anaerovibrio* sp. (Biddle et al., 2013). As nsCHO enter the cecum, amylolytic microorganisms rapidly proliferate and degrade starch. This increases lactate concentration in the cecum, which then serves as substrate for lactate-utilizing microorganisms, leading to proliferation of this group of bacteria. Illustrating the relationship between lactate-producing and lactate-utilizing microorganisms i*n vivo*, horses consuming 2 g starch·kg BW^{-1}·$meal^{-1}$ of oats (high starch) or 1 g starch·kg BW^{-1}·$meal^{-1}$ of oats (low starch) had increased proportions of fecal *Lactobacilli* and lactate-utilizing microorganisms compared to a forage only diet (Harlow et al., 2016). Simultaneously, the combined proportions of *Streptococcus* and *Enterococci* were reduced (Harlow et al., 2016). Moreau et al. (2014) also reported a positive relationship between lactate-utilizing *Veillonella* and starch-degrading microorganisms. Cecal populations of *Lactobacillus* and *Streptococcus* increased from 0.35% and 0.1% average relative abundance, respectively, in control horses to 27.78% and 15.0% when

corn starch was orally administered (Moreau et al., 2014). Administration of oral oligofructans also increased proportions of *Lactobacillus* (9.2%) and *Streptococcus* (0.6%) compared to horses assigned the control treatment (0.35 and 0.01, respectively; Moreau et al., 2014). As populations of *Lactobacillus* and *Streptococcus* increased, cecal populations of *Veillonella* were also greater in horses that received oral doses of oligofructans (28.8%) and cornstarch (6.7%) compared to control horses (0.35%; Moreau et al., 2014). This is likely due to the production of lactate by *Lactobacillus* and *Streptococcus* and a subsequent increase in *Veillonella*, which degrade lactate produced by amylolytic microorganisms.

Carbohydrate overload

Inclusion of > 2 g starch·kg BW^{-1}·$meal^{-1}$ exceeds the digestive and absorptive capacity of the small intestine, leading to starch entering the equine cecum (Potter et al., 1992). Undigested nsCHO that enter the equine cecum are rapidly degraded by amylolytic microorganisms, resulting in increased production of lactate and propionate (Willard et al., 1977). In horses that are not adjusted to large amounts of nsCHO entering the cecum, when such an influx occurs, production of lactate may exceed the capacity of lactate-utilizing microorganisms to remove lactate from the cecal environment. Lactic acid is 10 times more acidic than other VFA produced by cecal microorganisms (Nagaraja and Titgemeyer, 2007). As a result, accumulation of lactate leads to depressed cecal pH. If enough nsCHO enters the cecum following a single meal, an environment toxic to fibrolytic microorganisms develops. Warzecha et al. (2017) evaluated the immediate effect of an abrupt increase in dietary nsCHO (1.8 g nsCHO·kg BW^{-1}·$meal^{-1}$ in addition to *ad libitum* coastal Bermuda grass hay) on the equine cecal microbiome. Baseline diet consisted of *ad libitum* coastal Bermuda grass hay. Within 12 h of the dietary change, populations of cellulolytic microorganisms (*Ruminococcaceae*) decreased while populations of

saccharolytic (*Lactobacillaceae* and *Prevotella*) and lactate-utilizing microorganisms (*Anaerovibrio*) increased (Warzecha et al., 2017). Aligning with microbial population shifts, consumption of large amounts of nsCHO led to increased molar percentages of propionate and total VFA and tended to reduce pH. Cecal pH was < 6 in 2 horses, with horses exhibiting fevers and symptoms associated with acidosis; however, concentration of lactate was not measured (Warzecha et al., 2017). *In vitro* fermentation of starch, or starch with added lactate, by equine fecal inoculum reduced pH (7 vs. 4.5 to 6) at approximately h 18 (Biddle et al., 2013). This reduction in pH aligned with increased concentrations of *Streptococcus* (Biddle et al., 2013). *Lactobacillus*, a more acid-tolerant microorganism, peaked later during the fermentation process when growth of *Streptococcus* was limited (Biddle et al., 2013). Populations of the lactate-utilizers *Veillonella* and *Megasphera* began increasing at h 20 and continued to increase throughout the 48-h fermentation period (Biddle et al., 2013). In cattle, ruminal pH < 5 triggers onset of acute acidosis, resulting in cellular death of lactate-utilizing and fibrolytic microorganisms, thus triggering release of endotoxins (Nagaraja et al., 1978). While a pH associated with cecal acidosis in horses has yet to be defined, it is believed a similar mode of action occurs as concentration of endotoxins in blood are increased during episodes of starch-induced laminitis (Bailey et al., 2009). Development of 16S bacterial sequencing has allowed a greater understanding regarding impact of diet on microbial populations within the equine hindgut; however, metabolism of specific microorganisms and interactions between functional groups of microorganisms is still widely unknown.

Microbial sequencing

History of DNA sequencing

In 1952, Rosalind Franklin photographed DNA using X-ray technology, which would lead to the elucidation of the double helical structure of DNA by Watson and Crick in 1953 (Watson and Crick, 1953). It was only a few years later that DNA replication was made possible through the discovery of DNA polymerase (Bessman et al., 1958; Mullis, 1990). These advancements provided the foundation for RNA and DNA sequencing; however, it wasn't until 1965 that Holley et al. (1965a) discovered the first complete sequence of transfer RNA (tRNA) from *Saccharomyces cerevisiae* specific for alanine. The authors initially identified small fragments of the RNA which had been liberated by complete digestion via pancreatic ribonuclease and takadiastase ribonuclease T_1. Fragments were identified using chromatography on diethylaminoethyl (DEAE)-cellulose or DEAE-spandex and paper electrophoresis, commonly referred to as 2-D fractionation (Holley et al., 1965b). Authors then applied takadiastase ribonuclease T_1 to the tRNA to yield larger fragments (Holley et al., 1965a). These larger fragments allowed researchers to elucidate the order of nucleic bases. Simultaneously, Sanger et al. (1965) developed a similar 2-D fractionation method utilizing radioactive RNA, obtained by growing *E. coli* on media containing [^{32}P] phosphate. Partial digestion of *E. coli* was utilized to determine nucleotide sequence via 2-D fractionization followed by electrophoresis on DEAE-paper. Sequencing of RNA from various bacteria was then performed throughout the late 1960's via 2-D fractionation (Heather and Chain, 2016). In 1968, Wu and Kaiser published the base sequence of cohesive ends of bacteriophage λ by attaching radioactive nucleotides via DNA polymerase to the overhanging portion of the cohesive end (Wu and Kaiser, 1968). Shortly thereafter the entire genome of the cohesive ends was discovered (Wu and Taylor, 1971). This

process, however, required many chemicals and was slow, as only single nucleotides were added at a time. To reduce the number of steps and time required to sequence DNA, specific oligonucleotides were then used in conjunction with DNA polymerase to create complimentary strands of DNA (Sanger et al., 1973).

In the 1970's DNA sequencing was further advanced through the development of 2 methods: the "plus and minus" system and the chemical cleavage technique (Sanger and Coulson, 1975; Maxam and Gilbert, 1977). With the "plus and minus" system a synthetic oligonucleotide primer was extended and duplicated via DNA polymerase (Sanger and Coulson, 1975). During this initial step, nucleotides were radioactively labelled and random oligonucleotides of various lengths were yielded. Excess oligonucleotides were then removed. For the "plus" portion of the technique, the radioactively labelled oligonucleotide mixture was incubated with DNA polymerase and 1 triphosphate base. Mixtures were then separated via gel electrophoresis and the location of bands on the gel represented all positions of the added triphosphate base. Regarding the "minus" aspect, the random radioactive oligonucleotide mixture was separated into 4 samples. Each sample was then exposed to DNA polymerase again, but only 3 triphosphates were available, with a different triphosphate removed from each sample. In this step, synthesis proceeded as normal, until the missing triphosphate was required to continue building the chain. Synthesized chains were denatured, the complementary strand sequenced using gel electrophoresis and radioautography, and locations of the missing oligonucleotides were identified as the terminal parts of each sequence fragment. The distance between bands from the "minus" and "plus" oligonucleotide mixtures were analyzed together to determine any consecutive residues and elucidate the DNA sequence (Sanger and Coulson, 1975). The "plus and minus" technique was widely practiced and was utilized to sequence the first whole DNA

genome, belonging to bacteriophage phi X174 (Sanger et al., 1977a). The “plus and minus” method, however, required multiple DNA fragments of varying length to obtain an entire genome and required several replications for each DNA sequence (Hutchison III, 2007). Maxam and Gilbert’s chemical cleavage technique did not require DNA polymerase; instead, specific chemicals were added to radiolabeled DNA to cleave different base pairs. Once cleaved, the samples were read via gel electrophoresis to determine sequence of nucleotides (Maxam and Gilbert, 1977). While the chemical cleavage technique is considered by some to mark the beginning of 1st generation sequencing (Heather and Chain, 2016), it was chain termination, or the dideoxy technique, developed by Sanger et al. (1977b) that would become the basis of DNA sequencing.

The dideoxy technique was similar to the “plus and minus” method (Sanger and Coulson, 1975) but utilized analogues of deoxyribonucleotides (dNTP) and radiolabeled 2’,3’ dideoxynucleotides (ddNTP; Sanger et al., 1977b). There is no 3’ hydroxyl group on ddNTP, a bond is not formed with the next 5’ phosphate, and, thus, chain length cannot be further extended, and the chain is terminated (Sanger et al., 1977b; Heather and Chain, 2016). By supplying ddNTP in addition to the corresponding dNTP, ddNTP are randomly incorporated into the new chain, forming chains of varying length. To determine location of bases, 4 reactions are conducted in separate vessels, with each containing a different radiolabeled ddNTP. Following the reaction, gel electrophoresis is performed in parallel and nucleotide sequence can be identified (Sanger et al., 1977b). One of the primary advantages of the dideoxy method is that nucleotides within a chain appear as bands and upwards of 300 nucleotides can be read on a single gel (Sanger et al., 1977b), reducing the number of reactions necessary to obtain a complete

sequence compared to the “plus and minus” system (Sanger et al., 1977a). Dideoxy sequencing became the prominent sequencing method used for the next 3 decades.

In 1990, the Human Genome Project was initiated and several advancements were made in sequencing technology, ranging from automated systems that allowed multiple samples to be sequenced in tandem, replacing radiolabeled ddNTP with color-dyed ddNTP, and using capillary gel electrophoresis as opposed to slab gel electrophoresis (Heather and Chain, 2016; Branton and Deamer, 2019; Schloss et al., 2020). Following completion of the Human Genome Project in 2003, funding was awarded to scientists working to improve sequencing technology and reduce the associated cost. The machines and techniques developed following the Human Genome Project marked the start of NGS (Schloss et al., 2020).

Next generation sequencing

Next generation sequencing, also referred to as second generation sequencing, is characterized by the ability to sequence larger genomes, use of solid supports and parallel sequencing, and reduced cost (Slatko et al., 2018). There are 2 different approaches to NGS: sequence by hybridization and sequence by synthesis (Slatko et al., 2018). Sequence by hybridization was first made available in the early 1990’s (Sekar et al., 2005) and utilizes a known sequence of oligonucleotides, or probes (Khodakov et al., 2016). When a DNA fragment is exposed to marked probes, the portion of probes which are hybridized with the target DNA fragment can be used to elucidate sequence of the fragment (Drmanac et al., 2002). Probes for hybridization, however, are generally less than 100 nucleotides long and, thus, multiple hybridizing and washing cycles are required to obtain longer sequences of DNA (Sekar et al., 2005). Due to the limited length of probes, sequence by hybridization is most commonly used to determine mutations in known DNA sequences (Slatko et al., 2018). Sequence by hybridization

does not require amplification of DNA, which simplifies the sequencing process, but it may not yield adequate molecular sensitivity to be used for determining unknown DNA sequences on a large scale (Khodakov et al., 2016). Due to these shortcomings, sequence by hybridization is not utilized to the same extent as sequence by synthesis.

Methods used to sequence by synthesis are a continuation of Sanger's dideoxy method; however, dideoxy terminators are no longer used (Slatko et al., 2018). While there are a large variety of platforms and methods available for sequence by synthesis (Metzker, 2010; Slatko et al., 2018), the basic approach is similar: fragmentation of DNA molecules, PCR amplification, and generation of a template library (Metzker, 2010). The template library contains the fragmented DNA and allows sequences to be read. Specific methodology regarding reading of DNA sequences depends upon platform, the most common of which are 454 GenomeSequencer FLX, Ion PGM, and platforms created by Illumina.

The first machine designed for NGS was the 454 GenomeSequencer FLX developed by 454 Life Sciences in 2005 and later obtained by Roche Applied Science (Rothberg and Leamon, 2008). This platform was designed to sequence nucleotides through pyrosequencing, a form of sequencing by synthesis (Ahmadian et al., 2006). Pyrosequencing technology had been available since 1985 (Melamede, 1985); however, this sequencing method yielded multiple short reads, and thus it wasn't until improved processing technology was available in the early 2000's that large scale application of pyrosequencing was practical (Rothberg and Leamon, 2008). Using the 454 method, sample DNA is fragmented into chains of 400 to 700 base pairs (Slatko et al., 2018). The DNA is then denatured into single strands and adapter sequences are utilized to attach fragments of DNA to beads. Only 1 DNA strand should be attached to a single bead (Rothberg and Leamon, 2008). Beads are then placed into individual wells on a plate, which contains

approximately 1 million wells, and beads are subjected to emulsion PCR (Heather and Chain, 2016). Following amplification, DNA is denatured, beads carrying a single strand of DNA are placed into wells of a fiberoptic slide, and beads containing enzymes for pyrosequencing and dNTP are washed over the plate (Rothberg and Leamon, 2008; Heather and Chain, 2016). As DNA is synthesized, pyrophosphate is converted to luciferase, ultimately producing light (Nyrén and Lundin, 1985). Light emitted is then measured. In order to elucidate order of nucleotides, only 1 dNTP is incorporated at a time and the light generated is measured in real-time, with height of the peaks obtained corresponding to the number of nucleotides incorporated (Ronaghi, 2001; Rothberg and Leamon, 2008). All samples on a well can be analyzed in parallel, with each bead yielding reads between 400 to 500 base pairs long. These shorter reads performed in parallel removed the need for elongation of DNA and reduced error associated with chemical additives and premature chain termination (Zhou et al., 2010). Homopolymer regions, however, can lead to larger error rates and chain length can only be inferred based on light emission (Zhou et al., 2010). Due to limitations associated with 454 sequencing and competition from other parallel sequence machines, production of the 454 GenomeSequencer FLX was terminated, but similar technologies are still utilized (Slatko et al., 2018).

The Ion PGM platform from Ion Torrent was first made available in 2010 (Liu et al., 2012; Slatko et al., 2018). Similar to 454 pyrosequencing, the Ion PGM requires DNA to be fragmented and attached, or ligated, to adapter sequences. Upon ligation with adapter sequences, these DNA fragments become the DNA library and are attached to beads for amplification via emersion PCR (Bragg et al., 2013; Head et al., 2018). Following PCR, beads are placed into individual wells and dNTPs are washed over the wells, 1 at a time (Slatko et al., 2018). As nucleotides are added to each chain, hydrogen atoms are released, reducing the pH of the

solution, which is monitored by the Ion PGM (Liu et al., 2012) in real-time. The reduction in pH is doubled when 2 of the same nucleotides are incorporated sequentially. When large sections of chains are homopolymers, however, errors may occur (Slatko et al., 2018). Incorporation of a dNTP will only occur if it is complimentary to the next single nucleotide in the fragment; thus, by measuring changes in pH as each dNTP is washed over the wells, sequences of DNA fragments are elucidated (Liu et al., 2012).

The most widespread sequencing platforms were created by Illumina, with the HiSeq available in 2010 and the MiSeq in 2011 (Liu et al., 2012; Slatko et al., 2018; Schloss et al., 2020). Subsequent platforms included the NextSeq, iSeq, and MiniSeq. These follow the same basic protocols but differ in time spent analyzing data and level of throughput. As a result, application of the platforms differs, with the MiSeq being a common platform for sequencing bacteria. Following fragmentation of DNA and attachment of adaptors, DNA is subjected to bridge amplification. During bridge amplification, DNA with adaptor sequences is placed on a glass slide which contains a solid-phase primer that is complimentary to adaptor sequences on the DNA, binding both the 5' and 3' end, creating a 'bridge' structure (Khodakov et al., 2016; Slatko et al., 2018). Deoxyribose nucleic acid polymerase and all 4 oligonucleotides, with each nucleotide attached to a unique fluorescent label, are then washed over the glass slide (Yohe and Thyagarajan, 2017; Slatko et al., 2018). The fluorescently labelled nucleotides contain a reversible terminator, or a blocked 3' hydroxyl group, which prevents addition of subsequent nucleotides. Nucleotides that were not incorporated into the chain are then washed away and the machine captures a fluorescent image to identify which nucleotide was incorporated. The reversible terminator and fluorescent label are then cleaved from the added nucleotide, washed off the plate, and the process is repeated up to 300 times (Zhou et al., 2010; Slatko et al., 2018).

As oligonucleotides are added throughout the PCR process, clusters of clonal populations from the original DNA strands are formed, with each glass slide supporting millions of clusters (Slatko et al., 2018). A unique feature of bridge amplification is the ability to add nucleotides to both ends of the DNA strand, referred to as pair-end sequencing, which can improve final read alignment (Khodakov et al., 2016). The primary issue encountered with the Illumina platforms, however, is greater error rates; as additional nucleotides are added, residual fluorescent dye may interact with the machine (Zhou et al., 2010). The large number of clusters on each plate, however, help to elucidate the true nucleotide sequence and each sample is sequenced multiple times to increase sequencing depth (Slatko et al., 2018).

Data analyses

Data collected via high-throughput sequencing are then subjected to analyses. Specific packages, such as mothur and Quantitative Insights Into Microbial Ecology (Qiime; pronounced "chime"), are generally used to evaluate the large amount of data generated from high-throughput sequencing. These packages can be used to calculate whether sequences can be separated into groups (referred to as structure of data), identify differences between groups, and detect any correlation between structure of data and groups (Gloor and Reid, 2016).

mothur was first released in 2009 by Schloss et al. (2009) as a comprehensive software package for analyzing microbial data. Prior to mothur, several different tools were necessary to trim, deconvulate and align sequences, calculate distance between sequences, assign operational taxonomic unit (OTU's), produce rarefication curves, estimate species richness and diversity, and compare structure and microbial diversity between communities (Schloss et al., 2009). An additional drawback to earlier microbial analyses tools was the need to trim and align sequences through online services. With advancements in technology, characterizing bacterial communities

through sequencing the16S ribosomal RNA gene became common and longer sequence reads were obtained. As a result, data files became too large to be sent through the internet and the use of multiple software tools was rendered infeasible (Schloss et al., 2009). The development of mothur provided a platform to perform the most common microbial analyses without the need to trim and align sequences through online services (Schloss et al., 2009). Since 2009, there have been many improvements regarding the coding of mothur, including integration of several smaller software programs to account for chimeras and errors in sequencing (Schloss et al., 2020).

During sequencing, the quantity of sequenced fragments of DNA, referred to as sequencing depth, varies between samples. To balance sequencing depth across samples, data are normalized, or rarefied, so all samples have the same number of reads, or library size (Weiss et al., 2015; Willis, 2019). One method of normalizing data requires a threshold, or minimum, number of reads. Samples which have a number of reads below the threshold are removed from the dataset. When the number of reads in a sample exceeds the threshold, reads are randomly removed so each sample has a number of reads equal to the threshold (Weiss et al., 2015). This can result in large quantities of samples being excluded or inclusion of errors due to inadequate sequencing depth. To balance sequencing depth and greater inclusion of samples, rarefication curves may be utilized to compare sequencing depth to the expected diversity of samples (Weiss et al., 2015). Another means of data normalization is to identify the sample with the fewest reads and randomly remove reads from all other samples until sequencing depth is equal in all samples (Willis et al., 2019). Both approaches to normalization are accepted, though both involve loss of data. Other techniques to transform depth of sequencing, such as log functions, have been

employed, though they may over- or underestimate the quantity of zeros, or missing microorganisms, present in the data (Weiss et al., 2015).

To prepare sequences for identification, the command “make.contigs” is inserted into mothur to combine pair-end reads. Overlapping of the forward and reverse sequence aids in quality control and confidence in the generated contig. If either the forward or reverse sequence contains a missing base, or gap, while the other does not, then a quality score of the intact sequence must be greater than 25 for mothur to accept the single base as correct. Using the Phred algorithm, quality scores for individual bases are calculated using signal-to-noise ratios and intensity of light emitted when each base is read. Bases with greater quality scores are more likely to be correct, while bases with poorer quality scores are more likely to be errors (https://www.illumina.com/content/dam/illumina-marketing/documents/products/technote_understanding_quatliy_scores.pdf). If, in the overlapping section of a sequence, 2 bases do not match, the quality scores of both the forward and reverse sequences are compared. The quality score of 1 sequence must be at least 6 points greater than the other sequence to be considered accurate. If these cut-offs are not met the base is considered ambiguous. Contigs are then trimmed using “screen.seqs”, with starting sequences and ending sequences denoted. This step removes artifacts of PCR and any ambiguous sequences. Maximum length of sequences is typically determined automatically and is used to reduce error based on incorrect base pairing during PCR. Time required for analysis is reduced via the “unique.seqs” command which only keeps unique sequences for downstream analyses. Sequences are then identified using the selected reference database via the “align.seqs” command. Incorrect sequences (pre.cluster) and chimeras (chimera.vsearch) can then be identified and removed. Lastly, sequences are clustered

to form OTU's via the command "cluster.split", and "classify.otu" is used to identify OTU clusters (Kozich et al., 2013; https://www.mothur.org/wiki/miseq_sop/: Accessed 28 June 2021).

Diversity of microbial composition within a sample or a community is referred to as alpha diversity and can be a function of species richness, species evenness, or both (Willis, 2019). Species richness is defined as the number of species present, whereas evenness is the relative abundance of a species (Engelbrekston et al., 2010). There are various indices which have been developed to calculate alpha diversity, including Shannon diversity index, also referred to as Shannon's index and Shannon-Weiner's index, and Simpson's index. Both calculations account for richness and evenness of a microbial community. Shannon's index places greater importance on richness, whereas Simpson's index emphasizes evenness (Kim et al., 2017; Thukral, 2017). Similarly, Fisher's alpha diversity index is a logarithmic calculation of species richness (Fisher et al., 1943; Thukral, 2017). This calculation is considered to be independent of sample size and may be more sensitive than Shannon's index to microorganisms present in low abundance due to exclusion of species evenness in the equation (Hayer and Buzas, 2013). Observed OTU's is also utilized to characterize microbial diversity and is representative of species richness (Lemos et al., 2011). In an attempt to correct for errors in sequencing, OTU's are clustered based upon either 97% or 99% similarity of sequence (Schloss and Westcott, 2011). Chao1 and the Abundance-based Coverage Estimator (ACE) are non-parametric calculations used to determine species richness of a population, with emphasis on species found in low-abundance (Kim et al., 2017; Knight et al., 2018). Shannon's index, Simpson's index, Fisher's diversity, observed OTU's, Chao1, and ACE are quantitative and non-phylogenetic approaches to calculating alpha diversity. Faith's phylogenetic diversity (Faith's PD) is a quantitative assessment of alpha diversity which is the cumulative length of all branches on the phylogenetic

tree obtained from a microbial community (Faith, 1992). Use of both phylogenetic and non-phylogenetic approaches is beneficial as phylogenetic approaches factor in genetic evolution of the community whereas non-phylogenetic approaches only account for sequence output.

Diversity between samples is represented by beta diversity (Legendre et al., 2005). There are 2 types of beta diversity indices, qualitative and quantitative, which provide different information, allowing better understanding of the microbial community when both types are utilized (Sterling and Wilsey, 2001). The Unweighted Unifrac distance matrix is a qualitative index which utilizes phylogenetic data. The total branch lengths of the phylogenetic trees of communities being analyzed are calculated individually and then divided by the total branch length of both communities combined (Lozupone et al., 2007). In this way, the Unweighted Unifrac distance matrix can be used to determine differences in the phylogenetic diversity between communities, specifically microbial communities (Lozupone et al., 2007). The Bray-Curtis distance matrix is a quantitative, non-phylogenetic approach to calculate beta diversity (Tuomisto and Ruokolainen, 2006). Using the Bray-Curtis distance matrix, the population of microorganisms found in both samples are divided by the total number of microorganisms in both samples (Bray and Curtis, 1957). Beta diversity is generally displayed on Principle Coordinate Analysis (PCoA) plots (Tuomisto and Ruokolainen, 2006).

Sequences obtained from sequencing platforms must then be cross-referenced against databases of known microbial sequences for identification. Available databases include the EzBioCloud, BLAST through NCBI, EBI metagenomics, Greengenes, and SILVA, with SILVA being one of the more frequently used (Agnihotry et al., 2020) and comprehensive databases (Plummer et al., 2015). SILVA was first released in 2007 for the phylogenetic classification of small subunit (SSU) and large subunit (LSU) rRNAs present in bacteria, archaea, and eukarya

and has been updated several times since it was originally launched (Pruesse et al., 2007; Quast et al., 2013). SILVA 138, released in December 2019, contains 9,469,656 SSU sequences in the Parc dataset. The Parc sequence dataset is comprised of all sequences pertaining to the gene of interest that have a minimum alignment identity value of 50 and a minimum alignment quality of 40 (Quast et al., 2013; https://www.arb-silva.de/ documentation/release-138/). Identity value represents the total value of shared base pairs between a sequence and known reference sequences compared to the length of the reference sequence (Girgis et al., 2021; https://www.arb.de/act-turtorial). Alignment quality is a function of the identity value that represents similarity between a sequence and a reference sequence but is normalized and does not depend on sequence length (Pruesse et al., 2007; https://www.arb-silva.de/documentation/faqs/). Sequences containing less than 1,200 bases for bacteria or an alignment identity less than 50 are removed from the SSU Parc dataset to obtain the SSU Ref file. These are considered high-quality sequences (Quast et al., 2013). This file contains 983,534 bacterial sequences in SILVA 138. The SSU Ref non-redundant (NR) file in SILVA 138 contains 431,785 bacterial sequences. These sequences are obtained by subjecting SSU Ref sequences to a 99% identity criterion to remove identical sequences (https://www.arb-silva.de/documentation/release-138/). SILVA 138 integrates several taxonomic classification databases, including SILVA taxonomy, based on Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004; Yilmaz et al., 2013), NCBI, Genome Taxonomy Database, LPSN and UniEuk (https://www.arbsilva.de/documentation/ release-138/).

16S rRNA sequencing

The 16S gene is 1,600 base pairs in length and commonly utilized for sequencing, identifying, and evaluating lineage of bacterial microorganisms (Mignard and Flandrois, 2006;

Bukin et al., 2019). This gene contains highly conserved regions present in all bacterial microorganisms that are utilized to determine higher taxonomic classifications, while also consisting of 9 variable regions (V1 – V9) which are used to identify more specific taxonomic classification (Weisburg et al., 1991; Wang and Qian, 2009). With the shorter read length of sequences obtained via NGS, selection of the targeted variable region is important, as the conservative region does not provide adequate resolution to detect differences at lower taxonomic classifications. No single variable region, however, can identify all bacteria at all levels of classification, and, therefore, it is common to use ≥ 2 variable regions together. Of the variable regions, V3 and V4 are commonly targeted for bacterial identification. The V4 region is categorized as a class I region and is the most closely related variable region to full length sequences. The V4 region is best suited for studying phylogenetics at the phylum level when novel bacterial sequences may be encountered (Yang et al., 2016). The V3 region is noted as a class II region and is useful at identifying microorganisms to the family and genus level (Chakravorty et al., 2007; Yang et al., 2016; Bukin et al., 2019). The third class of variable regions includes V2, which is more useful for identifying species (Chakravorty et al., 2007). When comparing a combination of V2-V3 to V3-V4, Bukin et al. (2019) reported similar Shannon's and Simpson's alpha diversity indices, but the V2-V3 region provided greater Choa1 and ACE indices. Thus, there is greater identification of species with the V2-V3 region than the V3-V4 region (Bukin et al., 2019). The increased number of species identified led to a greater number of OTUs and different beta diversity when the V2-V3 region was used compared to the V3-V4 region (Bukin et al., 2019). Thus, the regions utilized will depend upon targeted level of taxonomic classification.

Bacterial culturing

The use of 16S sequencing is crucial for determining bacterial composition, but it does not yield information regarding function of microorganisms, particularly when novel sequences are obtained (Creevey et al., 2014). To elucidate specific roles of microorganisms, culturing methods are employed. In regards to microorganisms found within mammalian digestive tracts, either mixed culture or pure monoculture techniques can be utilized. Mixed cultures allow for study of synergistic relationships between microorganisms and cross-feeding, whereas monocultures allow better understanding of preferred substrates and end-products of fermentation of a specific microorganism (Krause et al., 2013). There are several options for media utilized in these cultures, including general-purpose media and selective media. General purpose media is utilized when culturing a wide variety of microorganisms; conversely, selective media is used target growth of a specific group of microorganisms, typically through addition of specific substrate, such as glucose for amylolytic bacteria (Dehority and Grubb, 1976). The addition of digestive fluid collected from the animal to culture media may enhance microbial growth due to additional growth factors present in digesta which cannot be duplicated in the laboratory. When culturing gastrointestinal microorganisms, it is essential to create an anerobic environment. This can be accomplished by flushing cultures with nitrogen to remove all oxygen, the use of a GasPak and a palladium catalyst, or conducting all culturing procedures within an anerobic environment (Hungate, 1969; Seip and Evans, 1980). Not all microorganisms, however, can be grown in a laboratory setting due to various factors including, but not limited to, pH, osmolarity, temperature, inappropriate or unknown nutrients and growth factors, and symbiotic relationships with other microorganisms (Stewart, 2012). Presently, it is estimated that approximately 20% of microorganisms in the rumen of cattle (Zehavi et al., 2018) and 23% of

microorganisms from the human gut (Lagier et al., 2018) can be cultured. Therefore, the role and behavior of microorganisms grown in culture may differ drastically from their natural environment. Thus, data obtained from *in vitro* culture techniques must be interpreted accordingly and drawing inferences regarding response or role of microorganisms *in vivo* based on data obtained via *in vitro* techniques should be restricted.

Summary

The equine gastrointestinal tract, particularly the hindgut, hosts a variety of anerobic microorganisms which degrade proteins, nsCHO, and sCHO. Prececal digestion of protein likely results in a N-limited environment throughout the equine hindgut. In order to maximize fermentation of sCHO, however, N – including BCFA – are necessary to meet microbial requirements for maintenance and proliferation. In conjunction with N, a supply of energy is necessary to maximize microbial efficiency. Both sCHO and nsCHO are degraded in the equine hindgut, yielding VFA. Volatile fatty acids then provide energy to microorganisms. Non-structural CHO, however, do not, from an evolutionary standpoint, comprise a large portion of the equine ration, but are rapidly fermented by microorganisms. As a result, feeding > 2 to 4 g starch·kg BW^{-1}·$meal^{-1}$ may lead to starch entering the cecum of the horse, resulting in dysbiosis due to rapid proliferation of amylolytic microorganisms and reduction in populations of fibrolytic microorganisms. While much is still unknown regarding the microbial ecosystem of the equine gastrointestinal tract, 16S bacterial sequencing has provided further insight into bacterial populations in the equine hindgut and the impact of diets on the microbial ecosystem.

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Chapter 6 - Effect of increasing levels of dietary starch on equine cecal microbiota

Abstract

Fluctuations in relative abundances of microorganisms in the equine hindgut have been associated with colic and hindgut disturbances, and, while equine diets contain varying ratios of forage:concentrate, little is known regarding effects of increasing dietary starch on the microbiome of the equine hindgut. Thus, an experiment was conducted with six cecally cannulated horses (524 ± 65.5 kg BW) to evaluate effects of increasing dietary starch on equine cecal microbiota. Starch was supplied via pelleted corn and increased by 0.5 g starch·kg BW^{-1}·$meal^{-1}$ every 7 d until horses received 3.5 g starch·kg BW^{-1}·$meal^{-1}$. Smooth bromegrass hay and water were offered *ad libitum*. Meals were fed every 6 h, starting at 0600 h. On d 7 of each period, cecal digesta were collected every 2 h for 12 h, with the h 0 collection occurring prior to the 0600 h feeding. Cecal samples obtained from all time points for a given level of dietary starch within an individual horse were pooled, DNA was extracted for PCR amplification of the 16S rRNA gene (V3 and V4 regions), and sequencing was performed using an Illumina MiSeq. mothur was utilized for clustering of features and operational taxonomical units (OTUs), and sequences were submitted to SILVA database for identification. Data were analyzed (SAS version 9.4) as a completely randomized design with fixed effect of treatment (g starch·kg BW^{-1}·$meal^{-1}$) and random effect of horse. Across treatments, Firmicutes was the most abundant phylum, followed by Bacteroidota. Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ elicited the greatest changes in microbiota, indicated by decreased ($P \leq 0.0469$) relative abundances (RA) of *Rikenellaceae, Prevotellaceae, RF16 group, Spirochaetaceae, Alloprevotella, Prevotella UCG-003, Prevotella UCG-004, RF16 group* genus, and *Treponema* compared to all other treatments.

Conversely, feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in increased ($P \leq 0.0045$) RA of *Christensenellaceae* and the *R-7 group* genus compared to all other treatments. If a horse presented with symptoms of colic, it was removed from the experiment. Data obtained when feeding 0.5, 1.0, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ were compared between horses that completed the trial and those removed using a covariate of 0 g starch·kg BW^{-1}·$meal^{-1}$. When consuming 0 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted had greater ($P \leq 0.00454$) RA of *Aeromonadales, Succinivibrionaceae,* and *Selenomonadaceae* compared to horses that were removed. When feeding 0.5 g starch·kg BW^{-1}·$meal^{-1}$, no differences in RA of taxa were detected between horses that persisted and horses that would later be removed. Horses that were removed had greater RA of *Colidextribacter* ($P = 0.0057$) compared to horses that persisted when feeding 1.0 g starch·kg BW^{-1}·$meal^{-1}$. When consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted had greater ($P \leq 0.0500$) RA of *Negativicutes, Acidaminococcales, Acidaminococcaceae, Phascolarctobacterium,* and *Ruminococcus* compared to horses that were removed. This is the one of the first reports describing effects of gradually increasing dietary starch on equine cecal microbiota *in vivo*. As well, this is the first report to compare cecal microbiota of horses tolerant of increasing dietary starch to those susceptible to colic in response to such dietary challenge.

Introduction

The horse evolved to consume forage almost continuously throughout the day. To degrade and utilize structural carbohydrates (sCHO) found in forages, the horse has an enlarged cecum and colon that facilitate microbial fermentation of sCHO to volatile fatty acids (VFA; Bergman, 1990). A horse at maintenance can meet up to 78% of its energy requirement through VFA, with 30% provided via microbial fermentation within the cecum and the rest provided through microbial fermentation in the colon (Glinsky et al., 1976; Vermorel et al., 1977). Performance horses, however, have increased energy requirements that often necessitate supplementation of energy. To meet increased energy requirements, managers often contradict the natural feeding behavior of horses by providing 1 to 3 feedings/d with varying ratios of forage:concentrate. Over-feeding starch may lead to colic, dysbiosis, and altered products of fermentation (Garner et al., 1978; Gonçalves et al., 2002; Crawford et al., 2007). Recommendations regarding maximum safe levels of dietary starch are largely based on the findings of Potter et al. (1992). They reported feeding > 3 to 4 g starch/kg body weight (BW)/meal exceeded the digestive and absorptive capabilities of the small intestine and led to starch flowing into the cecum. While Potter et al. (1992) suggested the presence of starch in the equine cecum was deleterious, they did not analyze impact of starch on the cecal environment. Another important consideration in making dietary recommendations is that pre-cecal digestibilities of grains vary; thus, the level at which starch overload occurs is expected to differ among grains (de Fombelle et al., 2004).

Development of next generation sequencing has allowed rapid characterization of microbial populations and has been utilized to study effects of diet on the microbiome of the rumen (Mignard and Flandrois, 2006; Janda and Abbott, 2007; Deng et al., 2008). Meanwhile

the microbiome and its responses to dietary alterations in the equine digestive tract remains relatively unexplored, as most data have been collected from euthanized animals where diet was often uncontrolled or have been generated using fecal samples that provide information restricted to the distal gastrointestinal tract. It is well documented that the fecal microbiome differs from the cecal microbiome of horses and thus is not a good model for the cecal environment (Dougal et al., 2012; Douthit et al., 2014; Fliegerova et al., 2016; Sorensen et al., 2019).

The purpose of this study was to characterize the effects of feeding increasing levels of starch (0 to 3.5 g starch·kg BW^{-1}·$meal^{-1}$) in an ascending dose titration design on the equine cecal microbiome.

Materials and methods

Animals

All animal procedures were approved by the Kansas State University Institutional Care and Use Committee. Six cecally cannulated Quarter horses (#7c, Bar Diamond, Parma, ID; Beard et al., 2011), 3 mares and 3 geldings aged 8 to 13 yr with average body weight (BW) of 524 ± 65.5 kg, were utilized in this study. Prior to initiation of the study, horses had *ad libitum* access to smooth bromegrass (brome) hay, water, and salt and were housed together in a dry lot pen.

Study design and treatments

To avoid deleterious effects associated with sudden increases in dietary starch, an ascending dose titration study design was implemented (Table 6.1). Rations consisted of brome hay, ration balancer, and pelleted corn (Table 6.2). The quantity of pelleted corn offered was increased every 7 d to provide an additional 0.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to the previous period, with treatments being 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 g starch·kg

BW^{-1}·$meal^{-1}$. Ration balancer was fed in conjunction with corn at a rate of 0.0125% (DM basis) BW/meal to meet NRC recommendations for vitamins, minerals, lysine, and protein for sedentary mature horses. Pelleted corn and ration balancer were offered every 6 h at 0600, 1200, 1800, and 2400 h with brome hay and water provided on an *ad libitum* basis.

Horses were weighed using a commercially available electronic livestock scale (EziWeigh 5i; Tru-Test Group, New Zealand) on d -14, 0, 7, 14, 21, 28, 35, and 42. These weights were used to calculate the amount of corn provided to each horse during the subsequent period. Horses were moved into individual stalls (3.05 m x 3.66 m) with rubber floor mats bedded with pine shavings on d -14. Horses could see one another throughout the experiment. On d 1, 8, 15, 22, 29, 36, and 43 dietary starch was increased by 0.125 g starch·kg BW^{-1}·$meal^{-1}$ until target level had been reached 24 h later. Cecal fluid was collected and pH measured prior to every meal throughout each 24-h transition period. If cecal pH dropped below 5.0, horses were considered at risk for cecal acidosis, additional starch was not offered, and horses were removed from the study. Horses were assigned pain scores (0 to 4) according to the Equine Comfort Assessment Scale (Blossom et al., 2007) and body temperature, heart rate, respiratory rate, and capillary refill time were recorded daily. If any animal exhibited abnormal health parameters, signs of intestinal discomfort, or a pain score ≥ 2, it was immediately removed from the study.

Sample collection

Approximately 100 mL cecal digesta were collected from each horse via gravity flow directly into 100-mL sterile specimen cups (Specimen Storage Containers, #14955117A; Fisher Scientific, Pittsburg, PA) every 2 h for 12 h relative to the 0600 h feeding on d 0, 7, 14, 21, 28, 35, 42, and 49. Hour 0 samples were collected immediately prior to the 0600 h feeding. Upon collection, pH was immediately measured using a portable pH probe (Thermo Orion Star A121;

Thermo Fisher Scientific Inc., Chelmsford, MA). Approximately 1.5 mL of unstrained cecal digesta were transferred in duplicate into microcentrifuge tubes and frozen at -20°C.

One of each duplicate sample collected from an individual horse throughout each 12-h collection period was pooled to create 1 representative sample for each horse on a given day. Briefly, individual 1.5-mL samples were thawed over ice and vortexed (Scientific Industries Vortex-Genie 2, Houston, TX) to mix contents. Within horse, 1 ± 0.01 g (as-sampled basis; Explorer Pro EP413 scale; Ohaus Corporation, Pine Brook, NJ) cecal digesta from each time point (h 0, 2, 4, 6, 8, 10, and 12) was transferred using sterile methods into a sterile conical tube (Nunc Conical Sterile Polypropylene Centrifuge Tubes; Thermo-Fisher Scientific, Waltham, MA), gently vortexed, and frozen at -20°C until DNA extraction.

Extraction of DNA

Extraction of DNA and subsequent sequencing was not performed on samples collected when feeding 3.5 g starch·kg BW^{-1}·$meal^{-1}$ as there were feed refusals and horses were not consuming the allotted starch during this feeding period. Deoxyribonucleic acid from all other pooled cecal samples was extracted using a commercially available kit (Quick-DNA™ Fecal/Soil Microbe Miniprep Kit; ZYMO Research Corporation, Orange, CA, USA). Manufacturer's instructions for DNA extraction were followed with the following adjustment: pooled cecal samples were placed into ZR BashingBead™ Lysis Tubes and homogenized for approximately 40 min using a table-top vortex mixer (02215365; Fisher Scientific, Atlanta, GA) equipped with a foam adapter, in lieu of the recommended 20 min on a table-top vortexer equipped with a 2-mL microcentrifuge tube attachment. Samples were centrifuged using a SORVALL® Pico microcentrifuge (75003238; Thermo Scientific, Waltham, MA). After extraction, DNA was placed into microcentrifuge tubes and immediately frozen at -80°C.

Polymerase chain reaction and DNA sequencing

Purity of DNA was analyzed via a Nanodrop8000 fluorometer (Thermo Fisher, Waltham, MA) and DNA concentration was measured using a Qubit (2.0; Invitrogen, Waltham, MA). Polymerase chain reaction (PCR) was performed on the V3 and V4 regions of the 16S bacterial gene using the forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG and the reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTAT AAGAGACAGGACTACHVGGGTATCTAATCC (Klindworth et al., 2013). Polymerase chain reaction, PCR clean-up, library quantification, and normalization were performed following the 16S Metagenomic Sequencing Library Preparation Guide (Illumina, 2013) at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS). Briefly, 5 ng/μL of genomic DNA were combined with 5 μL of 1 μM forward and reverse amplicon PCR primers and 12.5 μL 2x KAPA HiFi HotStart Ready Mix. Samples were placed in a thermo cycler and held at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Finally, samples were held at 72°C for 5 min for elongation. Primers were removed using AmPure XP beads. Index PCR and library building were performed utilizing the Nextera XT library prep kit (Illumina, San Diego, CA). Five microliters DNA; 5 μL Nextera XT Index Primers 1 and 2; 25 μL of 2x KAPA HiFi HotStart Ready Mix; and 10 μL PCR grade water were combined. Samples underwent PCR on a thermo cycler with the following protocol: 95°C for 3 min; 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; held at 72°C for 5 min. A second DNA purification was performed using AMPure XP beads prior to DNA quantification and normalization. Quantity of sequenced reads present in each sample was determined and the sample containing the fewest number of sequenced reads served as a threshold. Random reads were then removed from all other samples until library size of all samples was equal. Following

normalization, sample DNA was denatured with 10 μL 0.2 N NaOH and diluted with hybridization buffer. Sequencing of DNA was performed on an Illumina MiSeq system (Illumina Inc., San Diego, CA).

Raw sequences were pre-processed through mothur (https://mothur.org/) with removal of primers and adaptor sequences, as well as combining and decluttering of reads. SILVA database (Release 138; https://mothur.org/wiki/silva_reference_files/) was utilized for taxonomical classification and assignment of taxa to features (level of similarity). Sequences that could not be assigned at any specific taxonomic level were classified by the highest taxonomic level to which they could be assigned, followed by unclassified or uncultured.

Statistical analyses

Taxonomical data were analyzed utilizing the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) as a completely randomized design with random effect of horse. Degrees of freedom were modified utilizing the Kenward-Roger approximation. To test differences between microbial populations of horses that persisted throughout the study and those removed, post hoc analyses of relative abundance of known taxa were compared between the two groups of horses when feeding 0.5, 1.0, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$, with a covariate of relative abundance when horses consumed the control diet. If there was no effect of covariate, the covariate statement was removed and only average relative abundance of taxa between the two groups of horses was analyzed to avoid artifacts. Differences in average relative abundance of taxa between the 2 groups of horses are referred to as “effect of group”. Differences were declared at $P < 0.05$ and tendencies were considered at $0.05 < P < 0.10$. Taxa with differing average relative abundances between the two groups of horses were further analyzed, testing the effect of treatment as previously described, for horses that persisted and

those removed separately. Diversity indices were analyzed in R. Observed taxonomic units (OTU) were assigned in mothur and analyzed using the phyloseq package in R while the vegan package in R was utilized to analyze the Shannon diversity, Fisher's alpha diversity, Bray-Curtis dissimilarity, and Unifrac indices.

Results

Taxonomic composition

The number of unassigned bacteria increased as specificity of taxonomic classification increased, with none identified at the species level. Only sequences matching known bacteria for each taxonomic classification with ≥ 1% relative abundance are included in results and discussion herein. The most pronounced changes in equine cecal microbiota were observed when horses were fed 1.5 g starch·kg BW^{-1}·$meal^{-1}$, with populations of several taxa differing when feeding this concentration of starch compared to all other treatments.

Phyla

There were main effects of treatment on the phyla Firmicutes (Table 6.3; Fig. 6.1; $P < 0.0001$), Bacteroidota ($P < 0.0001$), Proteobacteria ($P = 0.0384$), Spirochaetota ($P = 0.0022$), Fibrobacteria ($P = 0.0027$), and Actinobacteriota ($P = 0.0004$). There was no effect of treatment on average relative abundance of Verrucomicrobiota ($P = 0.7362$). Firmicutes was the predominant phylum and feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to increased ($P < 0.0001$) average relative abundance of Firmicutes compared to other treatments. Feeding 2.0 g starch·kg BW^{-1}·$meal^{-1}$ resulted in greater ($P < 0.0078$) relative abundance of Firmicutes compared to 3.0 g starch·kg BW^{-1}·$meal^{-1}$.

Bacteroidota was the second most abundant phylum and, compared to all other treatments, 1.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in decreased proportions of the phylum

Bacteroidota ($P < 0.0001$). There were reduced proportions of Bacteroidota when horses consumed 2.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 ($P = 0.0072$), 1.0 ($P = 0.0303$), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0014$). Proportions of Bacteroidota tended to be reduced when feeding 2.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0722$). Average relative abundance of Bacteroidota was less when horses consumed 0.5 ($P = 0.0431$), 1.5 ($P \leq 0.0001$), and 2.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0014$), and tended to be less when horses consumed 1 ($P = 0.0874$) and 2.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0658$) compared to 3.0 g starch·kg BW^{-1}·$meal^{-1}$.

Relative abundance of Proteobacteria was greater when feeding 1.0 ($P \leq 0.0101$), 2.0 ($P \leq 0.0174$), and 2.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P \leq 0.0451$) than when horses were fed 0 and 1.5 g starch·kg BW^{-1}·$meal^{-1}$.

Relative abundance of Spirochaetota was reduced when horses consumed 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to all other treatments ($P \leq 0.0148$). Feeding 3.0 g starch·kg BW^{-1}·$meal^{-1}$ led to greater average relative abundance of Spirochaetota compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0352$) and tended to increase average relative abundance compared to 1.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0592$).

Horses consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ had reduced proportions of Fibrobacterota compared to 0 ($P < 0.0001$), 0.5 ($P = 0.0008$), 1.0 ($P = 0.0380$), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0339$). Feeding 1.0 ($P = 0.0175$), 2.0 ($P = 0.0091$), and 2.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0194$) resulted in reduced average relative abundance of Fibrobacterota compared to 0 g starch·kg BW^{-1}·$meal^{-1}$.

Average relative abundance of Actinobacteriota was greatest ($P \leq 0.0087$) when horses consumed 1.5 g starch·kg BW^{-1}·$meal^{-1}$. Feeding 2.0 g starch·kg BW^{-1}·$meal^{-1}$ resulted in reduced

average relative abundance of Actinobacteriota compared to 0 ($P = 0.0214$) and 0.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0176$).

Class, Order, Family

Effects of treatment on class, order, and family taxonomic classifications are presented in detail in tables 6.3, 6.4, and 6.5, respectively.

Genera

Within the Firmicutes phylum, there were main effects of treatment on *Blautia*, ($P = 0.0069$), *Ruminococcus* ($P < 0.0001$), *Anaerovibrio* ($P < 0.0001$), *Phascolarctobacterium* ($P = 0.0009$), *R-7 group* ($P = 0.0022$) *Lactobacillus* ($P = 0.0064$), and *Streptococcus* ($P = 0.0013$; Table 6.6). There were no effects of treatment on relative abundance of *Marvinbryantia* ($P = 0.3707$), *Colidextribacter* ($P = 0.1215$), or *Oscillospiraceae UCG-005* ($P = 0.3743$). Average relative abundances of *Blautia* ($P \leq 0.0131$) and *Ruminococcus* ($P \leq 0.0397$) were reduced when horses were consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to all other treatments. Horses consuming 0 g starch·kg BW^{-1}·$meal^{-1}$ had the greatest ($P \leq 0.0004$) proportion of *Ruminococcus.* Average relative abundance of *Ruminococcus* was greater when horses were consuming 0.5 ($P = 0.0129$) and 1.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0089$) compared to 2.0 g starch·kg BW^{-1}·$meal^{-1}$. There was greater average relative abundance of *Anaerovibrio* when feeding 2.0 and 2.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 ($P < 0.0001$), 0.5 ($P \leq 0.0043$), 1.0 ($P \leq 0.0097$), and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P \leq 0.0004$). Horses consuming 3.0 g starch·kg BW^{-1}·$meal^{-1}$ had greater relative abundance of *Anaerovibrio* compared to 0 ($P = 0.0035$) and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0121$). Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in reduced ($P \leq 0.0188$) relative abundance of *Phascolarctobacterium* compared to all other treatments. There were greater ($P \leq 0.0032$) proportions of *R-7 group* when horses were fed 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to

all other treatments. Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to increased ($P \leq 0.0031$) relative abundance of *Lactobacillus* compared to all other treatments except 2.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0589$). Relative abundance of *Streptococcus* was greater when horses were offered 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to all treatments ($P \leq 0.0052$) with exception of 2.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0942$). Feeding 2.0 g starch·kg BW^{-1}·$meal^{-1}$ led to increased relative abundance of *Streptococcus* compared to 0 ($P = 0.0128$) and 0.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0366$) and tended to increase average relative abundance of *Streptococcus* compared to 3.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0525$).

There were effects of treatment on genera *p-251-o5* ($P = 0.0080$), *RC9 gut group* ($P = 0.0012$), *RF16 group* ($P < 0.0001$), *Alloprevotella* ($P < 0.0001$), *Prevotellaceae UCG-003* ($P < 0.0001$), and *Prevotellaceae UCG-004* ($P = 0.0089$) within the Bacteroidota phylum. There was no effect ($P = 0.3637$) of treatment on *Prevotellaceae UCG-001.* Average relative abundance of *p-251-o5* was reduced when horses were consuming 1.5 g starch/kg BW meal compared to 0 ($P = 0.0012$), 0.5 ($P = 0.0005$), 1.0 ($P = 0.0030$), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0161$). Feeding 2.0 g starch·kg BW^{-1}·$meal^{-1}$ resulted in reduced ($P = 0.0373$) proportions of *p-251-o5* compared to 0.5 g starch·kg BW^{-1}·$meal^{-1}$. Horses consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ had reduced average relative abundance of *RC9 gut group* compared to all other treatments ($P \leq 0.0271$) except 1.0 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.0507$). Proportions of *RC9 gut group* were greater when horses were consuming 3.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to all other treatments ($P \leq 0.0106$) with exception of 2.5 g starch·kg BW^{-1}·$meal^{-1}$ ($P = 0.1724$). Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in less ($P \leq 0.0469$) average relative abundance of *RF16 group* compared to all other treatments. Horses consuming 3.0 g starch·kg BW^{-1}·$meal^{-1}$ had greater ($P \leq 0.0017$) average relative abundance of *RF16 group* compared to all other treatments except 2.5

g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.1021). Consumption of 2.5 g starch·kg BW^{-1}·$meal^{-1}$ resulted in increased average relative abundance of *RF16 group* compared to 0 (P = 0.0024), 0.5 (P = 0.0065), 1.0 (P = 0.0430), and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ (P < 0.0001). Horses consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ had reduced (P < 0.0001) proportions of *Alloprevotella* compared to all other treatments. Average relative abundance of *Alloprevotella* was greater when horses were consuming 0.5 (P = 0.0144), 1.0 (P = 0.0009), 2.0 (P = 0.0002), 2.5 (P < 0.0001), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (P < 0.0001) compared to 0 g starch·kg BW^{-1}·$meal^{-1}$. Horses consuming 0 g starch·kg BW^{-1}·$meal^{-1}$ had greater ($P \leq 0.0286$) average relative abundance of *Prevotellaceae UCG-003* compared to all treatments except 0.5 g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.0670). Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to the greatest reduction in ($P \leq 0.0436$) average relative abundance of *Prevotellaceae UCG-003.* Average relative abundance of *Prevotellaceae UCG-003* was less when horses were consuming 2.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 (P < 0.0001), 0.5 (P = 0.0018), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.0234). Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to reduced ($P \leq 0.0422$) proportions of *Prevotellaceae UCG-004* compared to all other treatments. Horses consuming 0.5 g starch·kg BW^{-1}·$meal^{-1}$ had reduced (P = 0.0362) average relative abundance of *Prevotellaceae UCG-004* compared to 0 g starch·kg BW^{-1}·$meal^{-1}$.

Within the Proteobacteria phylum, only an uncultured genus was detected with > 1% average relative abundance.

There was an effect of treatment on the genus *Treponema* (P = 0.0026) within the Spirochaetota phylum. Relative abundance of *Treponema* was reduced ($P \leq 0.0156$) when feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to all other treatments. Feeding 3.0 g starch·kg BW^{-1}·$meal^{-1}$ resulted in greater (P = 0.0388) average relative abundance of *Treponema*

compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ and tended to increase (P = 0.0620) average relative abundance compared to 1.0 g starch·kg BW^{-1}·$meal^{-1}$.

There was an effect of treatment on the genus *Fibrobacter* (P = 0.0032) within the Fibrobacterota phylum. Average relative abundance of *Fibrobacter* was reduced when horses consumed 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 (P = 0.0001), 0.5 (P = 0.0009), 1.0 (P = 0.0402), and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.0390).

Differences between horses that persisted and those removed

A total of three horses were removed from the experiment when feeding between 2.0 and 3.0 g starch·kg BW^{-1}·$meal^{-1}$. Two horses displayed symptoms of gastrointestinal discomfort and one horse refused to consume corn pellets. There was equal variance ($P \geq 0.1161$) between average relative abundance of all taxa of horses that persisted and those later removed when horses consumed 0 g starch·kg BW^{-1}·$meal^{-1}$. When horses were consuming 0 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted throughout the experiment had greater (P = 0.00454; Table 6.7) average relative abundance of the family *Selenomonadaceae* compared to horses that were removed. When feeding 0 g starch·kg BW^{-1}·$meal^{-1,}$ average relative abundance of the order *Aeromonadales* (P < 0.0001) and the family *Succinivibrionaceae* (P < 0.0001) was greater in horses that persisted compared to horses that were removed.

There were covariate effects when horses were consuming 0.5 g starch·kg BW^{-1}·$meal^{-1}$ on the *Christensenellales* order (P= 0.0089; Table 6.8), the *Christensenellaceae* family (P = 0.0089), and the *R-7* genus (P = 0.0062) between horses that persisted throughout the experiment and horses that were removed. When feeding 0.5 g starch·kg BW^{-1}·$meal^{-1}$, there were no differences ($P \geq 0.1030$) in average relative abundances of microbiota at any level of taxonomic classification between horses that persisted and those removed.

There were effects of covariate on phylum Proteobacteria (P = 0.0114; Table 6.9); classes *Bacilli* (P = 0.0346) and *Gammaproteobacteria* (P = 0.0075); orders *Clostridiales* (P = 0.0157) and *Lactobacillales* (P = 0.0281); families *Clostridiaceae* (P = 0.0157), *Lactobacillaceae* (P = 0.0447), *Streptococcaceae* (P = 0.0397), and *p-251-o5* (P = 0.0047); and genera *Oscillospiraceae UCG-005* (P = 0.0487), *Colidextribacter* (P = 0.0048), *R-7* (P = 0.0491), *Lactobacillus* (P = 0.0447), *Streptococcus* (P = 0.0382), and *p-251-o5 genus* (P = 0.0047) when horses were consuming 1.0 g starch·kg BW^{-1}·$meal^{-1}$. There was an effect of group on proportions of *Colidextribacter* when horses were consuming 1.0 g starch·kg BW^{-1}·$meal^{-1}$, whereby average relative abundance of *Colidextribacter* was greater (P = 0.0057) in horses that would later be removed compared to horses that persisted. Within the Bacteroidota phylum, there were main effects of group on proportions on the genera UCG-001 (P = 0.0493) *Alloprevotella* (P = 0.0478) and *p-251-o5* (P = 0.0156) when horses were consuming 1.0 g starch·kg BW^{-1}·$meal^{-1}$. When feeding 1.0 g starch·kg BW^{-1}·$meal^{-1}$, horses that would later be removed from the study had greater (P = 0.0493) proportions of *UCG-001* compared to horses that persisted. Average relative abundances of *Alloprevotella* (P = 0.0478) and *p-251-o5* (P = 0.0156) were greater in horses that persisted compared to horses that would later be removed when feeding 1.0 g starch·kg BW^{-1}·$meal^{-1}$.

There were effects of covariate on orders *Christensenellales* (P = 0.0017; Table 6.10) and *Erysipelotrichales* (P = 0.0200); families *Christensenellaceae* (P = 0.0017), *Erysipelotrichaceae* (P = 0.0065), and *RF16 group* (P = 0.0184); and genera *R-7* (P = 0.0032) and *RF16 group genus* (P = 0.0184) when horses were consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$. There were no effects of group on any of these taxa ($P \geq 0.3467$). When consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted throughout the experiment had greater average relative abundance of *Negativicutes*

(*P* = 0.0348), *Acidaminococcales* (*P* = 0.0500), *Acidaminococcaceae* (*P* = 0.0500), *Phascolarctobacterium* (*P* = 0.0500), and *Ruminococcus* (*P* = 0.0206) compared to horses that were later removed.

Effect of treatment on microbial populations that differed between groups

Within group, effects of treatment (0, 0.5, 1.0, 1.5, and 2.0 g starch·kg BW^{-1}·$meal^{-1}$; Fig. 6.6 to Fig. 6.15) on populations of taxa that were different between horses that persisted and those removed were evaluated. Feeding horses 1.5 g starch·kg BW^{-1}·$meal^{-1}$ was the final treatment all horses consumed; thus, only differences in average relative abundance for 0, 0.5, 1.0, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ treatments will be discussed. No differences were detected in average relative abundance of *Selenomonadaceae* when feeding 0.5, 1.0, or 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ in horses that persisted (*P* = 0.3415). Feeding 1 g starch·kg BW^{-1}·$meal^{-1}$, however, resulted in greater (*P* = 0.0495) average relative abundance of *Selenomonadaceae* compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ in horses that were later removed. Average relative abundance of *Aeromonadales* and its corresponding family, *Succinivibrionaceae*, did not differ ($P \leq 0.0805$) when horses that persisted were consuming 0.5, 1.0, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$. Horses that were removed had greater average relative abundance of *Aeromonadales* (*P* = 0.0300) and *Succinivibrionaceae* (*P* = 0.0300) when consuming 1 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$.

There was no difference in average relative abundance of *Colidextribacter* when feeding 1.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ for horses that persisted (*P* = 0.9557) or horses that were removed (*P* = 0.1863). Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to greater (*P* = 0.0080) average relative abundance of *Colidextribacter* compared to 0 g starch·kg

BW^{-1}·$meal^{-1}$ in horses that were removed from the experiment. This difference was not detected in horses that persisted (P = 0.6489).

Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ to horses that were removed from the study led to reduced average relative abundance of *Negativicutes* compared to feeding 0 g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.0169). There was no difference in average relative abundance of *Negativicutes* in horses that persisted when feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ (P = 0.9262). *Acidaminococcales* and *Acidaminococcaceae* proportions were reduced when horses that persisted (P = 0.0066) and horses that were removed (P = 0.0016) consumed 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$. Feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ led to reduced proportions of *Phascolarctobacterium* compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ in horses that persisted (P = 0.0061) and horses that were removed (P = 0.0016). Both horses that persisted (P < 0.0001) and horses that were removed (P = 0.0003) had reduced average relative abundance of *Ruminococcus* when consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$.

Alpha diversity

There were main effects of treatment on the Shannon diversity index (P < 0.0001), Fisher's alpha diversity (P = 0.00258) and observed taxonomic units (OTU; P = 0.00052).

Beta diversity

While we were unable to determine main effect of treatment for the Bray-Curtis dissimilarity or the Unweighted Unifrac index, differences between treatments were determined. There were no differences were detected between treatments with the Bray-Curtis dissimilarity or the Unweighted Unifrac index ($P \geq 0.1770$).

Discussion

The impact of dietary starch on the equine cecal microbiome is greatly dependent on prececal digestion and absorption of starch. The small intestine serves as the primary location of digestion of starch before fermentation in the hindgut; however, the extent of the small intestine's ability to digest and absorb starch varies with source and processing technique (Julliand et al., 2006; Rosenfeld and Austbø, 2009; de Fombelle et al., 2004). In general, there is greater prececal digestibility of starch from oats compared to starch from corn (Julliand et al., 2006; Rosenfeld and Austbø, 2009; de Fombelle et al., 2004). Additionally, increasing the surface area of grains via grinding increases prececal digestibility of starch (Julliand et al., 2006). Corn was selected as the source of starch in the present study as it is commonly included in equine diets and there is adequate literature available to allow for informed interpretation of results. In the current experiment, it was expected that the equine small intestine would have the capacity to digest and absorb ≤ 2 g starch·kg BW^{-1}·$meal^{-1}$ (Potter et al., 1992). Interestingly, feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ – an amount below this threshold - produced the greatest changes in the equine cecal microbiome, which will be discussed in more detail below. While horses were fed 1.5 g starch·kg BW^{-1}·$meal^{-1}$ through corn, they were also consuming starch in the ration balancer and hay. While hay consumption varied, on average, horses were consuming approximately 1.54 g starch·kg BW^{-1}·$meal^{-1}$ at this time when both hay and ration balancer were included, a level which is still below the 2 g starch·kg BW^{-1}·$meal^{-1}$ threshold proposed by Potter et al. (1992).

Firmicutes, the phyla comprising > 48% average relative abundance in the present study, has been identified by others as the most predominant throughout the equine hindgut (Dougal et al., 2014; Venable et al., 2017). In accordance with our observations, Firmicutes has been

reported as the most abundant phylum in equine fecal samples when horses were offered forage (Shepherd et al., 2012; Dougal et al., 2014; Fernandes et al., 2014) or forage and concentrate (Steelman et al., 2012; Dougal et al., 2014; Fernandes et al., 2014; Costa et al., 2015), as well as in equine cecal samples when horses were offered forage and concentrate (Jevit, 2016; Venable et al., 2017). Bacteroidota, previously referred to as Bacteroidetes (Whitman et al., 2018), has also been reported as the most predominant phylum in equine cecal (Warzecha et al., 2017; Sorensen et al., 2019) and fecal samples (Stewart et al., 2017). Authors reporting Bacteroidota in greater proportions compared to Firmicutes typically fed horses all-forage diets (Stewart et al., 2017; Sorensen et al., 2019; Su et al., 2020). Feeding high-grain diets increases populations of Firmicutes and reduces Bacteroidota in the rumen of cattle (Mao et al., 2013). While similar shifts were observed when feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$, the return to baseline values when feeding 3.0 g starch·kg BW^{-1}·$meal^{-1}$ for both Firmicutes and Bacteroidota was unexpected. This may have been due to a more neutral cecal pH in the present study (average 6.73) compared to that of Mao et al. (2013) who reported average ruminal pH of approximately 5.9 during the 12 h following feeding. A less acidic cecal pH may have allowed for more controlled proliferation of amylolytic microorganisms and continued proliferation of fibrolytic microorganisms, allowing the relative abundance of cecal microorganisms to stabilize and return to baseline values. It is important, however, to note phyla contain multiple microorganisms that utilize a variety of substrates, thus making interpretation of microbial data at the phyla level extremely challenging.

Microorganisms associated with degradation of starch, such as *Streptococcus*, *Lactobacillus*, *Phascolarctobacterium*, and *Succinivibrionaceae*, have been identified throughout the hindgut of healthy horses consuming concentrates (Costa et al., 2015). When cattle that are not adjusted to starch-rich diets are provided a large bolus of starch, both *Streptococcus* and

Lactobacillus rapidly proliferate, degrade starch, and produce large quantities of lactic acid, resulting in acidotic ruminal conditions (Russell and Hino, 1985; Coe et al., 1999). Similarly, it appears *Lactobacillus* and *Streptococcus* may contribute to cecal acidosis in horses. Garner et al. (1978) reported oral administration of corn starch at levels designed to induce cecal acidosis and laminitis in horses resulted in greater *Lactobacillus* proportions, while Milinovich et al. (2006) found increased proportions of *Streptococcus* sp. in the feces of horses immediately prior to the onset of laminitis. *Streptococcus* is the primary etiological microorganism responsible for lactic acidosis in fermentative chambers of horses and ruminants as it rapidly degrades starch, releasing organic acids at a rate that overwhelms the ability of other cecal microorganisms to utilize these acids (Biddle et al., 2006). Subsequently, pH is reduced. Botha et al. (2012) reported *L. equigenerosi*, a specie of *Lactobacillus* unique to the horse, can proliferate, and thus degrade substrate, in cultures with pH = 4. Therefore, reduced cecal pH may favor proliferation of *Lactobacillus* which could further reduce cecal pH as *Lactobacillus* continues to ferment starch. Meanwhile, it has been demonstrated that microorganisms responsible for degradation of pectin (Smart et al., 1964), hemicellulose (Morrison, 1976; Lowe et al., 1987), and cellulose (Hu et al., 2004; Sung et al., 2006) are pH dependent, with reduced activity when pH is < 6 in ruminants. In the present study, average relative abundances of *Lactobacillus* and *Streptococcus* were greatest when horses were consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ but returned to baseline values when horses were consuming 2.0 and 2.5 g starch·kg BW^{-1}·$meal^{-1}$. Interestingly, the most acidic cecal pH ($P < 0.05$) in our study was observed when horses were consuming 2.0 g starch·kg BW^{-1}·$meal^{-1}$, with average cecal pH for this treatment being 6.58 (Ochonski, 2021). This cecal pH is considered normal for the research horses utilized in this experiment. Therefore, cecal pH may not be the best indicator of shifts in microbial populations, specifically proportions of amylolytic

to fibrolytic microorganisms, within the equine cecum. It should also be noted that the cecal pH which marks the onset of equine cecal acidosis has yet to be characterized, and horses in the present study were gradually transitioned to rations with greater quantities of starch to allow gradual adaptation and to facilitate stabilization of microbial populations within the equine cecum.

As expected, there was an inverse relationship between average relative abundance of amylolytic and fibrolytic microorganisms in the present study. This is likely due to competition for nutrients. With plentiful availability of starch in the cecum, amylolytic microorganisms would be expected to proliferate at a greater rate than fibrolytic microorganisms. As a result, other nutrients necessary for microbial proliferation, such as N, may have been monopolized by amylolytic microorganisms, thus reducing resources available for proliferation of fibrolytic microorganisms. The genera *Fibrobacter* and *Ruminococcus*, and the family *Lachnospiraceae* ferment fibrous components of feed (Stewart et al., 1997). Others have reported *Fibrobacter* and *Ruminococcus* are prevalent in the equine hindgut (Julliand et al., 1999; Julliand and Grimm, 2017). In the present study *Ruminococcus* proportions in the equine cecum were reduced when feeding all treatments compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ ($P \leq 0.0004$), indicating predominantly cellulolytic species within the *Ruminococcus* genus (Miller and Wolin, 1995; Julliand et al., 1999) were present in these horses. This reduction in fibrolytic microorganisms is likely due to the greater cecal concentration of lactate when horses consumed 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ compared control ($P \leq 0.05$; Ochonski, 2021) as lactic acid accumulation inhibits growth of fibrolytic microorganisms (Morrison, 1976; Lowe et al., 1987; Hu et al., 2004; Sung et al., 2006). This theory is further supported by the fact that each horse removed from the study had cecal concentrations of lactate between 11.0 and 14.3 mM prior to

removal, whereas cecal concentrations of lactate of horses that persisted remained below 4.0 mM throughout the duration of the study (Ochonski, 2021).

The return to baseline proportions of *Fibrobacter* when horses were consuming 3.0 g starch·kg BW^{-1}·$meal^{-1}$ in the current experiment was unexpected. Not only had consumption of concentrate increased, but voluntary forage intake also declined throughout the experiment. Horses consuming 0 and 0.5 g starch·kg BW^{-1}·$meal^{-1}$ consumed approximately 2.5% BW of brome per day; however, when feeding > 2.5 g starch·kg BW^{-1}·$meal^{-1}$, horses consumed between 0.9% and 1.35% BW of brome daily (Ochonski, 2021). It is possible the return to baseline average relative abundance of *Fibrobacter* when horses were consuming 3.0 g starch·kg BW^{-1}·$meal^{-1}$ was due to the proliferation of amylolytic microorganisms that utilize intermediary products of fermentation of starch. A primary intermediate product of fermentation of starch is lactate, which, when accumulated, reduces cecal pH (Nagaraja and Titgemeyer, 2007). Another factor important to fermentation of fiber and proliferation of fibrolytic microorganisms is attachment of microorganisms to fiber molecules. *Treponema* aids in attachment of fibrolytic microorganisms to fiber molecules, stimulating fermentation of structural carbohydrates (Stanton and Canale-Parola, 1980; Stewart et al., 1997). Relative abundance of both *Fibrobacter* and *Treponema* were below baseline values when 2.5 g starch·kg BW^{-1}·$meal^{-1}$ was consumed, but there was a concurrent increase in the relative abundance of both microorganisms when 3.0 g starch·kg BW^{-1}·$meal^{-1}$ was offered. Perhaps greater proportions of *Treponema* stimulated growth of *Fibrobacter* microorganisms by increasing adherence of these microorganisms to fiber molecules.

The return of both amylolytic and fibrolytic microorganisms to baseline proportions as horses progressively consumed more starch was likely due to the involvement of microorganisms

that utilize intermediary products of fermentation of starch, such as lactate. Without the presence of such microorganisms, it is unlikely that fibrolytic microorganisms would be able to compete with amylolytic microorganisms which rapidly degrade substrate, producing large quantities of acid and limiting activity of fibrolytic enzymes (Morrison, 1976; Lowe et al., 1987; Hu et al., 2004; Sung et al., 2006). Within the equine core microbiome *Veillonellaceae* and *Anaerovibrio* are known to utilize lactate (Biddle et al., 2013). In our experiment, average relative abundance of *Veillonellaceae* was less than 1%, and thus not included in statistical analyses. Relative abundance of *Anaerovibrio* increased above baseline values when horses were consuming 2.0 and 2.5 g starch·kg BW^{-1}·$meal^{-1}$, though there were no differences detected in average relative abundances of *Anaerovibrio* when feeding 2.0, 2.5, or 3.0 g starch·kg BW^{-1}·$meal^{-1}$. It is possible that horses consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ were not yet acclimated to the level of lactic acid produced from fermentation of starch, which is supported by reduced ($P \leq 0.05$) average cecal pH of 6.73 when horses were consuming this treatment compared to 0, 0.5, and 1.0 g starch·kg BW^{-1}·$meal^{-1}$ (Ochonski, 2021). Cecal pH, however, continued to decline to 6.58 and lactic acid concentration within the equine cecum was greatest when horses were consuming 2.0 g starch·kg BW^{-1}·$meal^{-1}$ (Ochonski, 2021). As the experiment progressed and dietary consumption of starch continued to increase, increased average relative abundance of *Anaerovibrio* likely reflects continued production of lactic acid, but accumulation was prevented by these lactate-utilizing microbes. This is supported by the reduction of cecal lactic acid concentration when horses were consuming 2.5 and 3.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to 2.0 g starch·kg BW^{-1}·$meal^{-1}$ (Ochonski, 2021). Interestingly, there was no difference in average relative abundance of *Anaerovibrio* between horses that persisted and those removed, suggesting

other lactate-utilizing microorganisms may have played a more important role in regulating the equine cecal environment.

Another common intermediary product of fermentation of starch is succinate, which is also produced by lactate-utilizing microorganisms (Nakamura and Takahashi, 1971). Both *Succinivibrionaceae* and *Phascolarctobacterium* utilize succinate, and, therefore, average relative abundances of these microorganisms were expected to increase with greater quantities of dietary starch (Stewart et al., 1997; Indugu et al., 2017; Plaizier et al., 2017). There was not, however, a clear correlation between increasing dietary starch and increased proportions of these microbes in the current experiment.

While the overall effect of treatment regarding lactate and succinate-utilizing microorganisms may be inconsistent, horses that persisted throughout the experiment likely had greater ability to adapt to starch-rich diets due in part to greater average relative abundances of intermediate product-utilizing microorganisms when the experiment began and baseline values were obtained. Horses that persisted throughout the study had greater average relative abundances of succinate-utilizing microorganisms (*Aeromonadales, Succinivibrionaceae,* and *Selenomonadaceae*) when consuming 0 g starch·kg BW^{-1}·$meal^{-1}$ compared to horses that were later removed, It should be also be noted that average relative abundance of *Aeromonadales* and *Succinivibrionaceae* when feeding 0 g starch·kg BW^{-1}·$meal^{-1}$ was 0.01% in horses that persisted and 0.00% in horses that were removed. Additionally, when feeding 0 g starch·kg BW^{-1}·$meal^{-1}$ average relative abundance of *Selenomonadaceae* was 0.19% in horses that persisted compared to 0.12% in horses that were removed. Thus, physiologic impact of the difference in baseline average relative abundance of these taxa between horses that persisted and those removed may be limited.

Interestingly, horses that would later be removed had increased relative abundances when consuming 1.0 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ for

Aeromonadales, Succinivibrionaceae, and *Selenomonadaceae* ($P \leq 0.0495$), which may indicate an attempt by intermediate-utilizing microorganisms to attenuate effects of starch entering the cecum. Given the decline in proportions of these microorganisms when consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ it appears, however, that these succinate and lactate-utilizing microorganisms were unable to continue proliferating at a rate necessary to prevent accumulation of lactic acid. Interpreting results of microbial data at the family level, however, should done cautiously, as different genera within families, such as *Selenomondaceae,* have been demonstrated to utilize lactic and succinic acids, while other genera produce lactic acid. The primary genera contributing to average relative abundance of *Selenomodaceae* in the present study is *Anaervibrio,* a known lactic-acid utilizing microbe.

When feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$, the final dietary treatment tolerated by all horses, those that persisted had greater average relative abundance of the class *Negativicutes,* the order *Acidaminococcales,* the family *Acidaminococcaceae,* and the genus *Phascolarctobacterium* compared to horses that were later removed. *Phascolarctobacterium* and its higher-order taxonomic classifications utilize intermediate products produced during fermentation of starch, including lactic acid (Stewart et al., 1997), which should reduce accumulation of lactic acid. Moreover, horses that were removed had reduced average relative abundance of *Negativicutes* when consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$, whereas similar differences were not noted in horses that persisted. Interestingly, average relative abundances of *Acidaminococcales, Acidaminococcaceae,* and *Phascolarctobacterium* were reduced when feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared to 0 g starch·kg BW^{-1}·$meal^{-1}$ in both groups of horses. It is possible that the combined effect of unknown genera or genera present in <1% average relative abundance within *Negativicutes* may have been the driving factor for the effect of treatment within group for *Negativicutes*.

It is necessary to note that from a clinical standpoint 3 horses were removed for different reasons: 1 horse refused to consume the full allotment of corn, 1 horse displayed symptoms of gas colic, and 1 horse displayed symptoms of impaction colic. Therefore, other factors in addition to cecal concentrations of lactate may have contributed to removal of these horses. Factors impacting the ability of the cecal microbiome in horses to adapt to increased levels of dietary starch, however, are poorly understood. While a link has been identified between genetics and microbial populations in the human gut (Goodrich et al., 2014; Waters and Ley, 2019), all but 1 of the animals used in this study had similar pedigrees; therefore, we would expect the genetic makeup to be similar and thus an unlikely driver behind microbiome differences in this study.

Unfortunately, due to complications with compatibility of sequence data from mothur and programs necessary to analyze diversity indices (phyloseq and vegan packages in R), interpretation of the available data for alpha and beta diversities is not prudent. It is interesting there were strong main effects of treatment on alpha diversity, or diversity within an individual community, but no differences between treatments were detected for beta-diversity indices, which represent microbial populations between communities. However, as no complete statistical output was yielded for any diversity index, it is likely errors occurred during the analytical process, rendering this information invalid.

Conclusion

Firmicutes was the most dominant phylum in the cecum of horses in the present study. Following the adaption protocol utilized, feeding horses 1.5 g starch·kg BW^{-1}·$meal^{-1}$ 4 times a day had the most dramatic effect on cecal microbiota. There was increased relative abundance of amylolytic microorganisms with a corresponding reduction in fibrolytic microorganisms when

horses were consuming 1.5 g starch·kg BW^{-1}·$meal^{-1}$ compared 0, 0.5, 1.0, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$. The greatest level of dietary starch, supplied via corn pellets, that was well tolerated by all horses was 1.5 g starch·kg BW^{-1}·$meal^{-1}$

Some horses may innately be able to degrade greater quantities of dietary starch than others, whether that be in the foregut or the hindgut. When feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$, horses that persisted in the present study had greater proportions of *Phascolarctobacterium,* which utilizes intermediate products of fermentation of starch, compared to the 3 horses that were removed from the study. Reduction in average relative abundance of the lactic acid utilizing *Negativicutes* class when feeding 1.5 g starch·kg BW^{-1}·$meal^{-1}$ to horses that were later removed likely contributed to accumulation of lactic acid in the cecal environment of these horses. Meanwhile, greater stability of proportions of taxa within the cecal microbiome of horses that persisted indicates that the microbiome of these horses better regulated the deleterious effects associated with feeding starch-rich diets.

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Table 6.1. Summary of ascending dose titration design used to increase dietary starch in cecally cannulated horses*.

Period	Days	Added starch, g·kg BW^{-1}·$meal^{-1}$‡‡	Added starch, g·kg BW^{-1}·d^{-1}‡	Horses on trial◊
1	-14 to 0	0.0	0	6
2	1 to 7	0.5	2	6
3	8 to 14	1.0	4	6
4	15 to 21	1.5	6	6
5	22 to 28	2.0	8	5
6	29 to 35	2.5	10	4‡‡**
7	36 to 42	3.0	12	3
8	43 to 49	3.5†	14	3

*Throughout the experiment, all horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

‡‡Dietary starch was increased in each new feeding period by 0.125 g starch·kg BW^{-1}·$meal^{-1}$ until target level was reached 24 h into each period.

†Cecal microbiome was not analyzed as horses were not consuming the full 3.5 g starch·kg BW^{-1}·$meal^{-1}$.

‡Horses were fed every 6 h starting at 0600 h.

◊Any horse presenting with symptoms of colic was removed from study.

**One horse did not consume full treatment amount and therefore, cecal samples collected from this horse were not analyzed for characterization of the microbiome.

Table 6.2. Proximate analyses of smooth bromegrass hay, pelleted corn, and ration balancer*.

Item, %	Smooth bromegrass hay	Pelleted corn	Ration balancer†
Dry matter	91.6	91.5	89.7
Crude protein	12.3	8.8	19.8
Acid detergent fiber	36.2	3.5	27.6
Neutral detergent fiber	63.2	7.1	37.9
Starch	0.8	69.4	5.9
Crude fat	3.9	3.4	1.8
Calcium	0.41	0.02	3.07
Phosphorus	0.18	0.28	1.58

*Proximate analysis using wet chemistry (Dairy One Forage Lab, Ithaca, NY) and reported on a dry matter basis.
†Mixed and pelleted at the O. H. Kruse Feed Technology Innovation Center, Kansas State University, Manhattan, KS.

Table 6.3. Taxa that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when horses were consuming increasing levels of dietary starch.

Phylum	Treatment*								
Class, %	0	0.5	1.0	1.5	2.0	2.5	3.0	SEM	*P*-value‡
Firmicutes	53.98^{ab}	54.88^{ab}	53.87^{ab}	79.40^{c}	59.37^{b}	55.80^{ab}	48.92^{a}	3.9120	< 0.0001
Clostridia	41.92^{a}	40.63^{a}	37.62^{a}	40.11^{a}	36.89^{a}	37.36^{a}	36.38^{a}	3.7789	0.4498
Bacilli	9.75^{a}	11.69^{ab}	13.31^{ab}	37.91^{c}	18.68^{b}	12.85^{ab}	7.99^{a}	5.4314	< 0.0001
Negativicutes	2.27^{ab}	2.51^{bc}	2.90^{cd}	1.34^{a}	3.86^{de}	4.72^{e}	3.70^{cde}	0.7321	0.0003
Bacteroidota	38.96^{cd}	36.51^{c}	37.48^{cd}	16.68^{a}	32.17^{b}	36.46^{bc}	42.39^{e}	3.0698	< 0.0001
Bacteroidia	38.96^{cd}	36.51^{c}	37.48^{cd}	16.68^{a}	32.17^{b}	36.46^{bc}	42.39^{e}	3.0698	< 0.0001
Proteobacteria	0.37^{a}	2.07^{ab}	3.53^{b}	0.31^{a}	3.43^{b}	3.38^{b}	2.22^{ab}	1.5987	0.0384
Gammaproteobacteria	0.33^{a}	2.04^{ab}	3.48^{b}	0.30^{a}	3.37^{b}	3.31^{b}	2.09^{ab}	1.5987	0.0422
Spirochaetota	0.97^{b}	1.47^{bc}	1.08^{bc}	0.11^{a}	1.54^{bc}	1.38^{bc}	1.89^{c}	0.4588	0.0022
Spirochaetia	0.97^{b}	1.47^{bc}	1.07^{bc}	0.11^{a}	1.52^{bc}	1.37^{bc}	1.88^{c}	0.4592	0.0024
Fibrobacterota	2.51^{c}	2.06^{bc}	1.17^{b}	0.006^{a}	0.93^{ab}	0.84^{ab}	1.51^{bc}	0.7418	0.0027
Fibrobacteria	2.51^{c}	2.06^{bc}	1.17^{b}	0.006^{a}	0.93^{ab}	0.84^{ab}	1.51^{bc}	0.7418	0.0027
Actinobacteriota	1.24^{b}	1.26^{b}	0.84^{ab}	1.90^{c}	0.65^{a}	0.74^{ab}	0.76^{ab}	0.6815	0.0004
Verrucomicrobiota	0.78^{a}	0.88^{a}	1.06^{a}	1.15^{a}	1.22^{a}	0.79^{a}	1.12^{a}	0.4245	0.7362
Number of horses	6	6	6	6	5	3	3		

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

*Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

‡Main effect of treatment.

[a,b,c,d]Means without a common superscript in a given row differ, $P < 0.05$.

Table 6.4. Orders that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when horses were consuming increasing levels of dietary starch.

Class	Treatment*								
Order, %	0	0.5	1.0	1.5	2.0	2.5	3.0	SEM	*P*-value[‡]
Clostridia									
Lachnospirales	22.96[a]	23.91[a]	21.56[a]	22.51[a]	21.93[a]	20.79[a]	20.77[a]	2.5974	0.7609
Oscillospirales	12.47[c]	10.97[bc]	10.93[bc]	7.91[a]	8.11[a]	11.24[bc]	9.55[ab]	1.2055	< 0.0001
Christensenellales	3.96[a]	3.26[a]	2.70[a]	6.85[b]	2.80[a]	3.04[a]	2.95[a]	1.2958	0.0026
Clostridiales	0.09[a]	0.34[a]	0.69[ab]	1.75[cd]	2.62[d]	1.11[abc]	1.71[bcd]	0.5939	0.0001
Bacilli									
Lactobacillales	4.54[a]	6.42[ab]	9.19[ab]	29.49[c]	16.12[b]	9.71[ab]	5.01[ab]	7.7376	0.0003
Erysipelotrichales	4.48[ab]	4.65[ab]	3.64[a]	7.34[b]	2.63[a]	3.27[ab]	2.91[ab]	2.0984	0.2448
Negativicutes									
Acidaminococcales	2.09[a]	1.67[a]	1.93[a]	0.95[b]	1.66[a]	1.62[a]	1.86[a]	0.3068	0.0010
Veillonellales-Selenomonadales	0.18[a]	0.84[ab]	0.97[ab]	0.39[a]	2.21[cd]	3.12[d]	1.85[bc]	0.5892	< 0.0001
Bacteroidia									
Bacteroidales	37.81[cd]	35.54[bc]	36.73[cd]	16.26[a]	31.45[b]	35.80[bc]	41.37[d]	2.9583	< 0.0001
Gammaproteobacteria									
Aeromonadales	0.002[a]	1.74[ab]	3.16[b]	0.05[a]	3.06[b]	3.00[b]	1.62[ab]	1.6060	0.0454
Spirochaetia									
Spirochaetales	0.97[b]	1.47[bc]	1.07[bc]	0.11[a]	1.52[bc]	1.37[bc]	1.88[c]	0.4592	0.0024
Fibrobacteria									
Fibrobacterales	2.51[b]	2.06[bc]	1.17[b]	0.006[a]	0.93[ab]	0.84[ab]	1.51[bc]	0.7418	0.0027
Number of horses	6	6	6	6	5	3	3		

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[‡]Main effect of treatment.

[a,b,c,d]Means without a common superscript differ, $P < 0.05$.

Table 6.5. Families that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when horses were consuming increasing levels of dietary starch.

Order	Treatment*								
Family, %	0	0.5	1.0	1.5	2.0	2.5	3.0	SEM	*P*-value‡
Clostridiales									
Clostridiaceae	0.09^{a}	0.34^{a}	0.69^{ab}	1.75^{cd}	2.62^{d}	1.11^{abc}	1.71^{bcd}	0.5939	0.0001
Lachnospirales									
Lachnospiraceae	22.88^{a}	23.81^{a}	21.49^{a}	22.42^{a}	21.87^{a}	20.73^{a}	20.68^{a}	2.2938	0.7675
Oscillospirales									
Ruminococcaceae	4.92^{b}	3.53^{ab}	3.99^{ab}	2.54^{a}	3.31^{ab}	4.96^{b}	2.86^{a}	1.0811	0.0521
Oscillospiraceae	6.76^{b}	6.91^{b}	6.45^{b}	5.21^{ab}	4.44^{a}	5.92^{ab}	6.31^{ab}	1.3057	0.1613
Veillonellales-Selenomonadales									
Selenomonadaceae	0.15^{a}	0.82^{ab}	0.95^{ab}	0.37^{a}	2.19^{cd}	3.08^{d}	1.83^{bc}	0.5924	< 0.0001
Acidaminococcales									
Acidaminococcaceae	2.09^{b}	1.67^{b}	1.93^{b}	0.95^{a}	1.66^{b}	1.62^{b}	1.86^{b}	0.3068	0.0010
Christensenellales									
Christensenellaceae	3.96^{a}	3.26^{a}	2.70^{a}	6.85^{b}	2.80^{a}	3.04^{a}	2.95^{a}	1.2958	0.0026
Lactobacillales									
Lactobacillaceae	2.73^{a}	2.76^{a}	3.46^{a}	7.73^{b}	3.35^{a}	4.61^{ab}	2.45^{a}	1.8216	0.0066
Streptococcaceae	1.50^{a}	3.42^{a}	5.45^{ab}	19.36^{c}	12.36^{bc}	4.63^{ab}	2.29^{ab}	5.3814	0.0013
Erysipelotrichales									
Erysipelotrichaceae	2.97^{ab}	3.53^{ab}	2.77^{ab}	6.86^{b}	2.10^{a}	2.59^{ab}	2.46^{ab}	2.4760	0.2033
Bacteroidales									
Unclassified	6.31^{b}	5.72^{ab}	6.83^{b}	4.07^{a}	6.76^{b}	6.94^{b}	7.54^{b}	1.2969	0.0509
p-251-o5	10.07^{bc}	10.48^{c}	9.58^{bc}	5.24^{a}	7.41^{ab}	7.82^{abc}	9.44^{bc}	1.8907	0.0080
Rikenellaceae	1.54^{b}	1.59^{b}	1.55^{b}	0.76^{a}	1.59^{b}	1.96^{bc}	2.55^{c}	0.4470	0.0099
RF16 group	1.11^{b}	1.37^{b}	1.91^{b}	0.04^{a}	2.18^{bc}	3.29^{cd}	4.51^{d}	0.7183	< 0.0001
Prevotellaceae	18.32^{c}	16.10^{bc}	16.56^{c}	6.00^{a}	13.68^{b}	16.25^{bc}	17.64^{c}	1.1632	< 0.0001
Aeromonadales									
Succinivibrionaceae	0.002^{a}	1.74^{ab}	3.16^{b}	0.05^{a}	3.06^{b}	3.00^{b}	1.92^{ab}	1.6080	0.0454
Spirochaetales									
Spirochaetaceae	0.97^{b}	1.47^{bc}	1.07^{bc}	0.11^{a}	1.52^{bc}	1.37^{bc}	1.88^{c}	0.4592	0.0024

Fibrobacterales									
Fibrobacteraceae	2.51[b]	2.06[bc]	1.17[b]	0.006[a]	0.93[ab]	0.84[ab]	1.51[bc]	0.7418	0.0027
Number of horses	6	6	6	6	5	3	3		

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[‡]Main effect of treatment.

[a,b,c,d]Means without a common superscript differ, $P < 0.05$.

Table 6.6. Genera that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when horses were consuming increasing levels of dietary starch.

Order *Family*, % *Genus*, %	Treatment*							SEM	*P*-value‡
	0	0.5	1.0	1.5	2.0	2.5	3.0		
Lachnospirales									
Lachnospiraceae									
Marvinbryantia	1.60^{ab}	2.21^{b}	1.86^{ab}	2.10^{ab}	2.03^{ab}	1.89^{ab}	1.33^{a}	0.4879	0.3707
Blautia	2.83^{b}	2.35^{b}	2.62^{b}	1.48^{a}	2.37^{b}	2.79^{b}	2.66^{b}	0.4456	0.0069
Unclassified	14.73^{a}	15.71^{a}	14.18^{a}	14.90^{a}	13.65^{a}	13.00^{a}	13.85^{a}	1.8188	0.6534
Oscillospirales									
Ruminococcaceae									
Ruminococcus	3.52^{d}	2.22^{c}	2.27^{c}	0.56^{a}	1.36^{b}	1.50^{bc}	1.39^{b}	0.4240	< 0.0001
Unclassified	0.71^{a}	0.82^{a}	1.23^{a}	1.52^{a}	1.60^{ab}	3.14^{b}	0.87^{a}	0.8859	0.0968
Oscillospiraceae									
Colidextribacter	1.34^{abc}	1.31^{abc}	1.55^{bc}	0.65^{a}	0.87^{ab}	1.22^{abc}	1.85^{c}	0.5141	0.1215
UCG-005	3.56^{ab}	4.04^{b}	3.50^{ab}	3.30^{ab}	2.41^{a}	3.31^{ab}	2.85^{ab}	0.9137	0.3743
Veillonellales-Selenomonadales									
Selenomonadaceae									
Anaerovibrio	0.08^{a}	0.76^{ab}	0.91^{ab}	0.35^{a}	2.16^{c}	2.99^{c}	1.77^{bc}	0.5930	< 0.0001
Acidaminococcales									
Acidaminococcaceae									
Phascolarctobacterium	2.08^{b}	1.67^{b}	1.92^{b}	0.93^{a}	1.65^{b}	1.62^{b}	1.86^{b}	0.3061	0.0009
Christensenellales									
Christensenellaceae									
R-7	3.72^{a}	3.10^{a}	2.55^{a}	6.63^{b}	2.71^{a}	2.97^{a}	2.86^{a}	1.2432	0.0022
Lactobacillales									
Lactobacillaceae									
Lactobacillus	2.73^{a}	2.76^{a}	3.46^{a}	7.70^{b}	3.35^{a}	4.61^{ab}	2.45^{a}	1.8082	0.0064
Streptococcaceae									
Streptococcus	1.47^{a}	3.38^{a}	5.41^{ab}	19.17^{c}	12.22^{bc}	4.59^{ab}	2.27^{ab}	5.3263	0.0013
Erysipelotrichales									

Erysipelotrichaceae									
Unclassified	2.18^{a}	3.04^{ab}	2.25^{a}	6.25^{b}	1.74^{a}	2.16^{ab}	2.06^{ab}	2.5174	0.2438
Bacteroidales									
Unclassified									
Unclassified	6.31^{b}	5.72^{ab}	6.83^{b}	4.07^{a}	6.76^{b}	6.94^{b}	7.54^{b}	1.2969	0.0509
p-251-o5									
p-251-o5 genus	10.07^{bc}	10.48^{c}	9.58^{bc}	5.24^{a}	7.41^{ab}	7.82^{abc}	9.44^{bc}	1.8907	0.0080
Rikenellaceae									
RC-9 gut-group	1.18^{bc}	1.22^{bc}	1.11^{ab}	0.62^{a}	1.36^{bc}	1.74^{cd}	2.21^{d}	0.3345	0.0012
RF16 group									
RF16 group	1.11^{b}	1.37^{b}	1.91^{b}	0.04^{a}	2.18^{bc}	3.29^{cd}	4.51^{d}	0.7183	< 0.0001
Prevotellaceae									
Alloprevotella	1.20^{b}	1.61^{c}	1.79^{c}	0.19^{a}	1.91^{c}	2.47^{d}	2.62^{d}	0.2195	< 0.0001
Unclassified	4.98^{c}	4.30^{bc}	5.04^{c}	1.56^{a}	3.36^{b}	4.09^{bc}	4.04^{bc}	0.7975	< 0.0001
UCG-001	4.35^{b}	3.90^{ab}	4.06^{ab}	2.70^{a}	2.94^{ab}	3.66^{ab}	3.89^{ab}	1.0875	0.3637
UCG-003	4.03^{e}	3.28^{de}	2.23^{bc}	0.49^{a}	2.06^{bc}	1.54^{b}	2.88^{cd}	0.5500	< 0.0001
UCG-004	2.84^{c}	1.78^{b}	2.12^{bc}	0.76^{a}	2.25^{bc}	2.64^{bc}	2.38^{bc}	0.6696	0.0089
Aeromonadales									
Succinivibrionaceae									
Uncultured	0.00^{a}	1.69^{ab}	3.13^{b}	0.05^{a}	1.54^{ab}	2.88^{b}	1.11^{ab}	1.1917	0.0096
Spirochaetales									
Spirochaetaceae									
Treponema	0.96^{b}	1.46^{bc}	1.06^{bc}	0.11^{a}	1.52^{bc}	1.35^{bc}	1.86^{c}	0.4587	0.0026
Fibrobacterales									
Fibrobacteraceae									
Fibrobacter	2.43^{d}	2.00^{cd}	1.14^{bc}	0.004^{a}	0.83^{ab}	0.80^{abc}	1.45^{bcd}	0.7345	0.0032
Number of horses	6	6	6	6	5	3	3		

[1]Collected every 2 h for 12 h relative to the 0600 h. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·meal^{-1}) supplied via pelleted corn and provided in 4 meals/d.

[‡]Main effect of treatment.

[a,b,c,d]Means without a common superscript differ, $P < 0.05$.

Table 6.7. Differences in taxa populations that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when feeding 0 g starch·kg BW^{-1}·meal^{-1}‡ between horses that persisted and those removed from the experiment.

	Group†			*P*-value	
	Persisted	Removed	MSE◊	G*	Levene's*
Phylum, %					
Class, %					
Order, %					
Family, %					
Genus, %					
Species, %					
Firmicutes	53.00	54.96	5.440	0.6819	0.3090
Clostridia	40.90	42.93	4.445	0.6058	0.1272
Clostridiales	0.04	0.13	0.123	0.3987	0.1491
Clostridiaceae	0.04	0.13	0.123	0.3987	0.1491
Lachnospirales	22.95	22.97	2.039	0.9879	0.1794
Lachnospiraceae	22.88	22.89	1.990	0.9938	0.1822
Unclassified	14.70	14.77	0.651	0.9086	0.1464
Blautia	2.82	2.85	0.464	0.9456	0.3063
Marvinbryantia	1.67	1.53	0.392	0.6797	0.3593
Oscillospirales	12.49	12.44	1.501	0.9282	0.3581
Oscillospiraceae	6.50	7.02	0.872	0.5007	0.1173
UCG-005	3.47	3.65	0.402	0.6117	0.5526
Colidextribacter	1.11	1.57	0.526	0.3388	0.1517
Ruminococcaceae	5.23	4.62	0.876	0.4360	0.1677
Unclassified	0.78	0.64	0.284	0.5847	0.5679
Ruminococcus	3.55	3.48	0.631	0.9014	0.2839
Christensenellales	3.14	4.77	1.860	0.3438	0.1172
Christensenellaceae	3.14	4.77	1.860	0.3438	0.1172
R-7	2.88	4.55	1.854	0.3308	0.1172
Negativicutes	1.98	2.55	0.493	0.2307	0.2058
Acidaminococcales	1.76	2.41	0.527	0.2035	0.2587
Acidaminococcaceae	1.76	2.41	0.527	0.2035	0.2587
Phascolarctobacterium	1.75	2.41	0.529	0.2025	0.2554

Veillonellales-Selenomonadales	0.22	0.14	0.056	0.1310	0.1546
Selenomonadaceae	0.19[a]	0.12[b]	0.030	0.0454	0.2498
Anaerovibrio	0.09	0.07	0.036	0.5528	0.1612
Bacilli	10.08	9.42	3.217	0.8155	0.1243
Lactobacillales	5.12	3.97	3.493	0.7075	0.1715
Lactobacillaceae	3.59	1.86	2.377	0.4231	0.1648
Lactobacillus	3.59	1.86	2.377	0.4231	0.1648
Streptococcaceae	1.16	1.83	0.987	0.4510	0.4406
Streptococcus	1.14	1.80	0.977	0.4524	0.4620
Erysipelotrichales	4.15	4.80	1.163	0.5331	0.1373
Erysipelotrichaceae	2.51	3.42	1.349	0.4526	0.1342
Unclassified	1.69	2.66	1.018	0.3088	0.1259
Bacteroidota	39.70	38.22	3.465	0.6283	0.9964
Bacteroidia	39.70	38.22	3.456	0.6283	0.9964
Bacteroidales	38.46	37.16	3.252	0.6517	0.5542
Unclassified	6.38	6.24	2.219	0.9395	0.5726
Unclassified	6.38	6.24	2.219	0.9395	0.5726
RF16 group	1.35	0.86	0.509	0.3086	0.1605
RF16 group	1.35	0.86	0.509	0.3086	0.1605
Prevotellaceae	17.17	19.48	1.914	0.2131	0.2157
Unclassified	4.42	5.53	1.416	0.3933	0.9203
UCG-001	3.18	5.51	1.363	0.1043	0.1350
UCG-003	5.01	3.05	0.875	0.0512	0.2342
UCG-004	2.26	3.42	1.751	0.4636	0.1281
Alloprevotella	1.37	1.02	0.360	0.3067	0.1230
p-251-o5	11.34	8.81	1.988	0.1931	0.7171
p-251-o5 genus	11.34	8.81	1.988	0.1931	0.7171
Rikenellaceae	1.84	1.24	0.591	0.2812	0.6890
RC-9 gut-group	1.24	1.12	0.321	0.6648	0.1353
Proteobacteria	0.34	0.39	0.356	0.8702	0.1673
Gammaproteobacteria	0.30	0.36	0.356	0.8388	0.1801

Aeromonadales	0.01[a]	0.00[b]	0.0003	< 0.0001	0.1161
Succinivibrionaceae	0.01[a]	0.00[b]	0.0003	< 0.0001	0.1161
Uncultured	0	0	0	NE[⁂]	NE[⁂]
Spirochaetota	1.12	0.82	0.237	0.1961	0.9248
Spirochaetia	1.12	0.82	0.237	0.1961	0.9248
Spirochaetales	1.12	0.82	0.237	0.1961	0.9248
Spirochaetaceae	1.12	0.82	0.237	0.1961	0.9248
Treponema	1.11	0.81	0.240	0.2031	0.9163
Fibrobacterota	2.54	2.49	1.566	0.9717	0.1627
Fibrobacteria	2.54	2.49	1.566	0.9717	0.1627
Fibrobacterales	2.54	2.49	1.566	0.9717	0.1627
Fibrobacteraceae	2.54	2.49	1.566	0.9717	0.1627
Fibrobacter	2.48	2.39	1.527	0.9447	0.1653
Actinobacteria	1.14	1.34	0.197	0.2716	0.1793
Verrucomicrobiota	0.87	0.70	0.323	0.5608	0.6782

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[‡]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and 0 g starch·kg BW^{-1}·$meal^{-1}$ supplied via pelleted corn and provided in 4 meals/d.

[†]Taxonomical data were divided into 2 groups post hoc, data collected from horses that persisted and consumed up to 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (n = 3) and data collected from horses that were removed from the study (n = 3) due to symptoms consistent with colic or a refusal to consume the dietary treatment.

[◊]Mean squared error.

[*]Main effect of group.

[‡]Levene's test for equal variance, $P > 0.05$.

[⁂]Not estimable.

[a,b]Means within a row with different superscripts differ, $P \leq 0.05$.

Table 6.8. Differences in taxa populations that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when feeding 0.5 g starch·kg BW^{-1}·meal^{-1}‡ between horses that persisted and those removed from the experiment.

	Group†			*P*-value	
	Persisted	Removed	MSE◊	G*	C*
Phylum, %					
Class, %					
Order, %					
Family, %					
Genus, %					
Species, %					
Firmicutes	51.48	58.27	8.871	0.4019	0.1319
Clostridia	37.61	43.66	5.849	0.2741	0.0785
Clostridiales	0.15	0.53	0.335	0.2341	0.2121
Clostridiaceae	0.15	0.53	0.335	0.2341	0.2121
Lachnospirales	22.67	25.16	3.752	0.4617	0.1311
Lachnospiraceae	22.59	25.04	3.685	0.4610	0.1355
Unclassified	15.07	16.36	2.926	0.6184	0.1794
Blautia	2.46	2.25	0.279	0.3973	0.7190
Marvinbryantia	1.92	2.49	0.678	0.3577	0.1828
Oscillospirales	10.42	11.21	1.338	0.3744	0.9830
Oscillospiraceae	6.13	7.68	1.627	0.3078	0.2058
UCG-005	3.17	4.92	1.475	0.2198	0.3810
Colidextribacter	1.49	1.14	0.209	0.1030	0.0900
Ruminococcaceae	3.79	3.26	1.106	0.5875	0.3865
Unclassified	0.85	0.80	0.308	0.8359	0.7566
Ruminococcus	2.46	1.97	0.944	0.5602	0.4722
Christensenellales	2.96	3.56	0.475	0.2633	0.0089
Christensenellaceae	2.96	3.56	0.475	0.2633	0.0089
R-7	2.82	3.39	0.422	0.2430	0.0062
Negativicutes	2.51	2.52	0.548	0.9852	0.2649
Acidaminococcales	1.58	1.77	0.344	0.5500	0.6864
Acidaminococcaceae	1.58	1.77	0.344	0.5500	0.6864
Phascolarctobacterium	1.58	1.76	0.346	0.5464	0.6964

Veillonellales-Selenomonadales	0.93	0.75	0.539	0.7081	0.2291
Selenomonadaceae	0.90	0.74	0.530	0.7202	0.3545
Anaerovibrio	0.82	0.70	0.499	0.7705	0.1846
Bacilli	11.32	12.05	4.481	0.8524	0.2675
Lactobacillales	7.03	5.80	3.743	0.7074	0.1826
Lactobacillaceae	2.36	3.16	2.191	0.6791	0.8163
Lactobacillus	2.36	3.16	2.191	0.6791	0.8163
Streptococcaceae	4.48	2.36	3.090	0.4477	0.0529
Streptococcus	4.43	2.33	3.067	0.4485	0.0538
Erysipelotrichales	3.59	5.70	1.770	0.2169	0.1127
Erysipelotrichaceae	2.52	4.54	1.847	0.2511	0.0804
Unclassified	2.25	2.25	0	NE[⁂]	< 0.0001
Bacteroidota	38.69	34.32	5.053	0.3489	0.0992
Bacteroidia	38.69	34.32	5.053	0.3489	0.0992
Bacteroidales	37.64	33.45	4.668	0.3336	0.1158
Unclassified	5.87	5.57	1.275	0.7851	0.2450
Unclassified	5.87	5.57	1.725	0.7851	0.2450
RF16 group	1.67	1.07	0.725	0.3693	0.2439
RF16 group	1.67	1.07	0.725	0.3693	0.2439
Prevotellaceae	17.31	14.88	2.996	0.3771	0.5447
Unclassified	4.85	3.76	0.587	0.0863	0.8251
UCG-001	2.72	5.07	1.107	0.0602	0.3822
UCG-003	4.22	2.35	1.127	0.1121	0.3970
UCG-004	2.13	1.43	0.682	0.2806	0.3292
Alloprevotella	1.83	1.39	0.472	0.3198	0.1666
p-251-o5	10.59	10.36	1.583	0.8688	0.6050
p-251-o5 genus	10.59	10.36	1.583	0.8688	0.6050
Rikenellaceae	1.95	1.24	0.944	0.4122	0.9910
RC-9 gut-group	1.55	0.89	0.920	0.4285	0.5236
Proteobacteria	2.72	1.42	3.003	0.6239	0.6441
Gammaproteobacteria	2.69	1.39	2.999	0.6231	0.6162

Aeromonadales	2.30	1.18	2.859	0.6570	0.0502
Succinivibrionaceae	2.30	1.18	2.860	0.6566	0.0503
Uncultured	2.28	1.11	2.815	0.6363	NE[‡‡]
Spirochaetota	1.77	1.18	0.759	0.3956	0.6378
Spirochaetia	1.76	1.17	0.756	0.3928	0.6441
Spirochaetales	1.76	1.17	0.756	0.3928	0.6441
Spirochaetaceae	1.76	1.17	0.756	0.3928	0.6441
Treponema	1.76	1.16	0.750	0.3848	0.6295
Fibrobacterota	2.55	1.57	1.474	0.4585	0.7672
Fibrobacteria	2.55	1.57	1.474	0.4585	0.7672
Fibrobacterales	2.55	1.57	1.474	0.4585	0.7672
Fibrobacteraceae	2.55	1.57	1.474	0.4585	0.7672
Fibrobacter	2.49	1.50	1.416	0.4366	0.8571
Actinobacteria	1.01	1.51	0.354	0.1583	0.1957
Verrucomicrobiota	0.90	0.85	0.215	0.7979	0.7747

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[‡]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and 0.5 g starch·kg BW^{-1}·$meal^{-1}$ supplied via pelleted corn and provided in 4 meals/d.
[†]Taxonomical data were divided into 2 groups post hoc, data collected from horses that persisted and consumed up to 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (n = 3) and data collected from horses that were removed from the study (n = 3) due to symptoms consistent with colic or a refusal to consume the dietary treatment.
[◊]Mean squared error.
[*]Main effect of group.
[‡]Effect of covariate (0 g starch·kg BW^{-1}·$meal^{-1}$).
[‡‡]Not estimable.
[a,b]Means within a row with different superscripts differ, $P \leq 0.05$.

Table 6.9. Differences in taxa populations that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when feeding 1.0 g starch·kg BW^{-1}·meal^{-1}‡ between horses that persisted and those removed from the experiment.

	Group†			*P*-value	
	Persisted	Removed	MSE◊	G*	C*
Phylum, %					
Class, %					
Order, %					
Family, %					
Genus, %					
Species, %					
Firmicutes	52.55	55.18	2.579	0.2797	0.7209
Clostridia	36.80	38.45	5.655	0.7385	0.2758
Clostridiales	0.59	0.79	0.328	0.5628	0.0157
Clostridiaceae	0.59	0.79	0.328	0.5628	0.0157
Lachnospirales	21.44	21.68	3.461	0.9382	0.7897
Lachnospiraceae	21.39	21.58	3.470	0.9480	0.7917
Unclassified	14.46	13.89	2.788	0.8130	0.6515
Blautia	2.66	2.57	0.667	0.8680	0.6701
Marvinbryantia	1.57	2.15	0.511	0.2361	0.1558
Oscillospirales	11.14	10.72	1.450	0.7347	0.2328
Oscillospiraceae	6.26	6.65	1.035	0.6641	0.1119
UCG-005	3.93	3.08	0.477	0.1253	0.0487
Colidextribacter	1.24[a]	1.87[b]	0.096	0.0057	0.0048
Ruminococcaceae	4.40	3.57	1.169	0.4814	0.6629
Unclassified	1.51	0.96	0.397	0.1979	0.0239
Ruminococcus	2.22	2.32	0.911	0.6849	0.3144
Christensenellales	2.40	3.01	0.969	0.4801	0.0622
Christensenellaceae	2.40	3.01	0.969	0.4801	0.0622
R-7	2.62	2.48	0.525	0.7871	0.0491
Negativicutes	2.69	3.11	0.792	0.5536	0.8963
Acidaminococcales	1.77	2.10	0.318	0.2648	0.1746
Acidaminococcaceae	1.77	2.10	0.318	0.2648	0.1746
Phascolarctobacterium	1.76	2.09	0.323	0.2725	0.1776

Veillonellales-Selenomonadales	0.93	1.01	0.655	0.8890	0.6051
Selenomonadaceae	0.91	0.99	0.653	0.8849	0.2495
Anaerovibrio	0.86	0.97	0.643	0.8434	0.9689
Bacilli	12.67	13.94	1.894	0.4749	0.0346
Lactobacillales	8.70	9.69	2.177	0.6238	0.0281
Lactobacillaceae	3.22	3.71	1.120	0.6619	0.0447
Lactobacillus	3.22	3.71	1.121	0.6607	0.0447
Streptococcaceae	6.41	4.50	1.992	0.3590	0.0397
Streptococcus	6.36	4.46	1.953	0.3515	0.0382
Erysipelotrichales	3.20	4.08	1.243	0.4317	0.5293
Erysipelotrichaceae	2.20	3.35	1.113	0.2768	0.4569
Unclassified	2.25	2.25	0.000	NE[*,**]	< 0.0001
Bacteroidota	37.62	37.35	1.342	0.8140	0.6185
Bacteroidia	37.62	37.35	1.342	0.8140	0.6185
Bacteroidales	36.79	36.67	1.509	0.9316	0.7152
Unclassified	5.84	7.81	1.613	0.2102	0.8979
Unclassified	5.84	7.81	1.613	0.2102	0.8979
RF16 group	2.61	1.22	0.664	0.0623	0.9216
RF16 group	2.61	1.22	0.664	0.0623	0.9216
Prevotellaceae	16.36	16.76	1.538	0.7694	0.2055
Unclassified	5.24	4.85	1.278	0.7270	0.7208
UCG-001	2.74[a]	5.38[b]	1.159	0.0493	0.3109
UCG-003	2.75	1.72	0.554	0.0842	0.0508
UCG-004	2.15	2.10	0.435	0.9041	0.5783
Alloprevotella	2.00[a]	1.58[b]	0.183	0.0478	0.5842
p-251-o5	11.01	8.16	0.554	0.0156	0.0047
p-251-o5 genus	11.01	8.16	0.554	0.0156	0.0047
Rikenellaceae	2.05	1.04	0.543	0.0848	0.6212
RC-9 gut-group	1.51	0.71	0.491	0.1144	0.6382
Proteobacteria	4.33	2.74	0.795	0.0924	0.0114
Gammaproteobacteria	4.29	2.67	0.692	0.0647	0.0075

Aeromonadales	3.69	2.62	2.151	0.5769	0.8822
Succinivibrionaceae	3.69	2.62	2.151	0.5769	0.8822
Uncultured	3.66	2.60	2.136	0.5791	NE[⁂]
Spirochaetota	1.30	0.85	0.469	0.3100	0.1165
Spirochaetia	1.29	0.85	0.466	0.3121	0.1188
Spirochaetales	1.29	0.85	0.466	0.3121	0.1188
Spirochaetaceae	1.29	0.85	0.466	0.3121	0.1188
Treponema	1.27	0.84	0.463	0.3198	0.1213
Fibrobacterota	1.73	0.60	0.881	0.1895	0.7925
Fibrobacteria	1.73	0.60	0.881	0.1895	0.7925
Fibrobacterales	1.73	0.60	0.881	0.1895	0.7925
Fibrobacteraceae	1.73	0.60	0.881	0.1895	0.7925
Fibrobacter	1.71	0.56	0.878	0.1851	0.8227
Actinobacteria	0.77	0.90	0.278	0.6007	0.2277
Verrucomicrobiota	0.87	1.24	0.345	0.2667	0.6648

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[‡]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and 1.0 g starch·kg BW^{-1}·$meal^{-1}$ supplied via pelleted corn and provided in 4 meals/d.

[†]Taxonomical data were divided into 2 groups post hoc, data collected from horses that persisted and consumed up to 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (n = 3) and data collected from horses that were removed from the study (n = 3) due to symptoms consistent with colic or a refusal to consume the dietary treatment.

[◊]Mean squared error.

[*]Main effect of group.

[⁑]Effect of covariate (0 g starch·kg BW^{-1}·$meal^{-1}$).

[⁂]Not estimable.

[a,b]Means within a row with different superscripts differ, $P \leq 0.05$.

Table 6.10. Differences in taxa populations that comprised ≥ 1% average relative abundance in equine cecal digesta[1] when feeding 1.5 g starch·kg BW^{-1}·meal^{-1}‡ between horses that persisted and those removed from the experiment.

	Group†			*P*-value	
	Persisted	Removed	MSE◊	G*	C*
Phylum, %					
Class, %					
Order, %					
Family, %					
Genus, %					
Species, %					
Firmicutes	75.29	83.51	7.074	0.2275	0.7978
Clostridia	39.47	40.76	10.727	0.8894	0.1493
Clostridiales	1.61	1.88	1.288	0.8108	0.1243
Clostridiaceae	1.61	1.88	1.288	0.8108	0.1243
Lachnospirales	23.08	21.95	5.809	0.8225	0.2121
Lachnospiraceae	22.98	21.86	5.834	0.8260	0.2095
Unclassified	15.27	14.53	3.275	0.7963	0.8789
Blautia	1.74	1.21	0.753	0.4365	0.5049
Marvinbryantia	2.45	1.74	0.898	0.3910	0.5060
Oscillospirales	8.68	7.13	2.589	0.5051	0.2593
Oscillospiraceae	6.61	3.82	2.597	0.2587	0.3822
UCG-005	4.18	2.42	1.573	0.2415	0.8975
Colidextribacter	0.86	0.43	0.671	0.4792	0.8865
Ruminococcaceae	1.91	3.17	0.733	0.1044	0.1245
Unclassified	0.72	2.32	0.604	0.0314	0.6455
Ruminococcus	0.93[a]	0.19[b]	0.245	0.0206	0.3466
Christensenellales	7.12	6.57	0.949	0.5762	0.0017
Christensenellaceae	7.12	6.57	0.949	0.5762	0.0017
R-7	6.96	6.31	1.131	0.5808	0.0032
Negativicutes	1.91[a]	0.77[b]	0.443	0.0348	0.4601
Acidaminococcales	1.26[a]	0.63[b]	0.277	0.0500	0.1209
Acidaminococcaceae	1.26[a]	0.63[b]	0.277	0.0500	0.1209
Phascolarctobacterium	1.24[a]	0.62[b]	0.273	0.0500	0.1156

Veillonellales-Selenomonadales	0.64	0.14	0.267	0.0806	0.1255
Selenomonadaceae	0.61	0.13	0.276	0.0967	0.2263
Anaerovibrio	0.58	0.11	0.271	0.1020	0.5512
Bacilli	33.88	41.95	13.636	0.5090	0.3166
Lactobacillales	28.55	30.44	19.506	0.9112	0.7077
Lactobacillaceae	6.68	8.77	3.887	0.5477	0.7194
Lactobacillus	6.68	8.71	3.831	0.5523	0.7203
Streptococcaceae	20.66	18.06	14.863	0.8412	0.8988
Streptococcus	20.43	17.91	14.704	0.8438	0.9042
Erysipelotrichales	6.30	8.39	3.510	0.5406	0.0200
Erysipelotrichaceae	6.26	7.47	2.456	0.6184	0.0065
Unclassified	6.25	6.25	0.000	NE[*,**]	< 0.0001
Bacteroidota	20.98	12.39	7.802	0.2491	0.4900
Bacteroidia	20.98	12.39	7.802	0.2491	0.4900
Bacteroidales	20.24	12.28	7.592	0.2684	0.3997
Unclassified	4.26	3.88	3.229	0.8947	0.2739
Unclassified	4.26	3.88	3.229	0.8947	0.2739
RF16 group	0.05	0.03	0.014	0.3467	0.0184
RF16 group	0.05	0.03	0.014	0.3467	0.0184
Prevotellaceae	7.09	4.92	2.286	0.3096	0.7547
Unclassified	1.86	1.27	1.017	0.5180	0.8517
UCG-001	3.31	2.07	1.027	0.2130	0.5557
UCG-003	0.43	0.55	0.330	0.7019	0.1375
UCG-004	0.98	0.54	0.559	0.3984	0.4758
Alloprevotella	0.22	0.15	0.129	0.5402	0.1186
p-251-o5	7.77	2.71	2.918	0.1009	0.7108
p-251-o5 genus	7.77	2.71	2.918	0.1009	0.7108
Rikenellaceae	0.98	0.55	0.468	0.3232	0.3873
RC-9 gut-group	0.80	0.44	0.442	0.3706	0.6562
Proteobacteria	0.54	0.08	0.337	0.1681	0.5024
Gammaproteobacteria	0.54	0.07	0.334	0.1645	0.4620

Aeromonadales	0.06	0.05	0.027	0.6957	0.4527
Succinivibrionaceae	0.06	0.05	0.027	0.6957	0.4527
Uncultured	0.05	0.04	0.035	0.8679	NE[⁂]
Spirochaetota	0.10	0.12	0.074	0.7061	0.8330
Spirochaetia	0.10	0.12	0.073	0.6820	0.8372
Spirochaetales	0.10	0.12	0.073	0.6820	0.8372
Spirochaetaceae	0.10	0.12	0.073	0.6820	0.8372
Treponema	0.10	0.12	0.072	0.6581	0.8273
Fibrobacterota	0.004	0.007	0.009	0.7322	0.5842
Fibrobacteria	0.004	0.007	0.009	0.7322	0.5842
Fibrobacterales	0.004	0.007	0.009	0.7322	0.5842
Fibrobacteraceae	0.004	0.007	0.009	0.7322	0.5842
Fibrobacter	0.004	0.0005	0.006	0.9479	0.6101
Actinobacteria	1.60	2.21	1.153	0.5482	0.3913
Verrucomicrobiota	1.06	1.24	1.159	0.8562	0.1142

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[‡]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and 1.5 g starch·kg BW^{-1}·$meal^{-1}$ supplied via pelleted corn and provided in 4 meals/d.

[†]Taxonomical data were divided into 2 groups post hoc, data collected from horses that persisted and consumed up to 3.0 g starch·kg BW^{-1}·$meal^{-1}$ (n = 3) and data collected from horses that were removed from the study (n = 3) due to symptoms consistent with colic or a refusal to consume the dietary treatment.

[◊]Mean squared error.

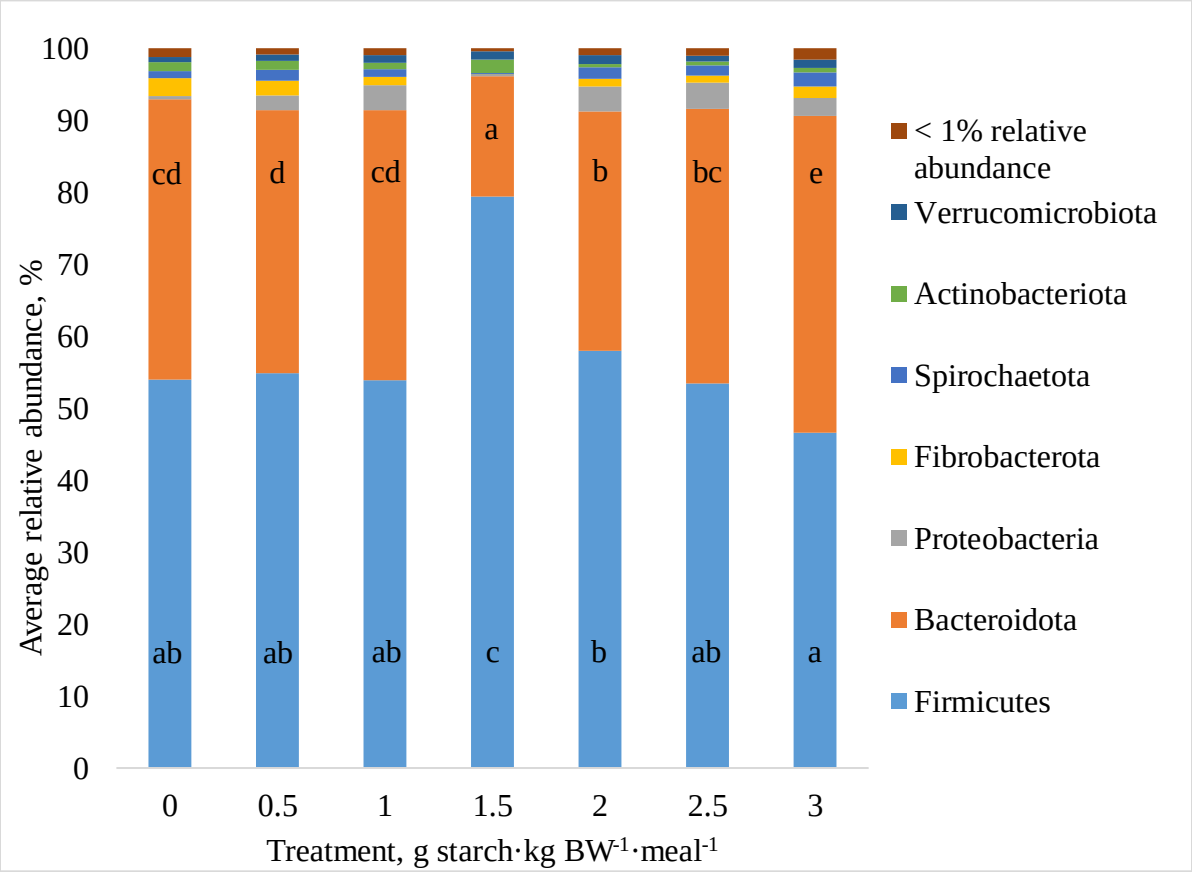
[*]Main effect of group.

[⁑]Effect of covariate (0 g starch·kg BW^{-1}·$meal^{-1}$).

[⁂]Not estimable.

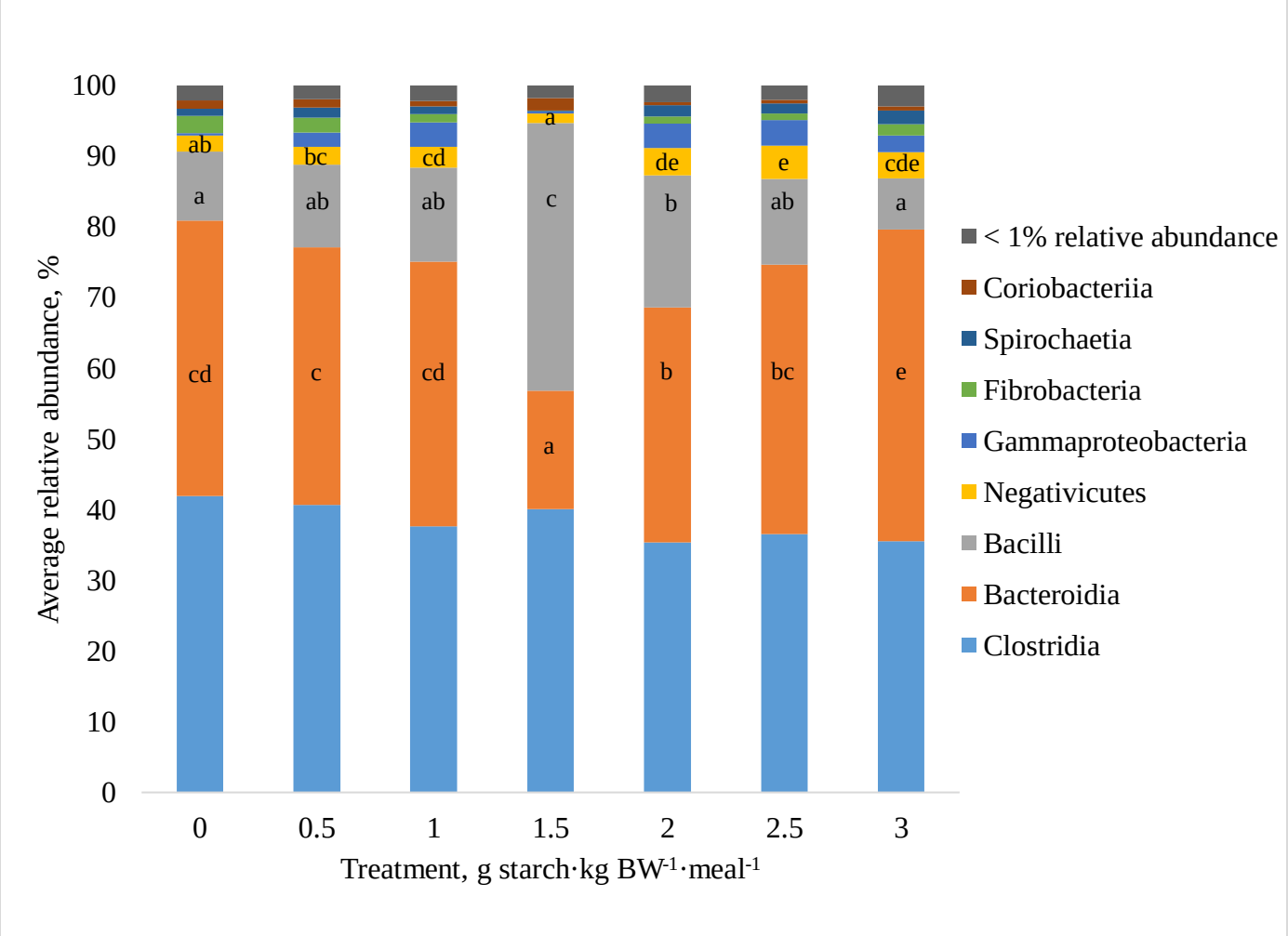
[a,b]Means within a row with different superscripts differ, $P \leq 0.05$.

Figure 6.1. Average relative abundance of bacterial phyla in equine cecal digesta[1] when horses were fed increasing levels of dietary starch[*].



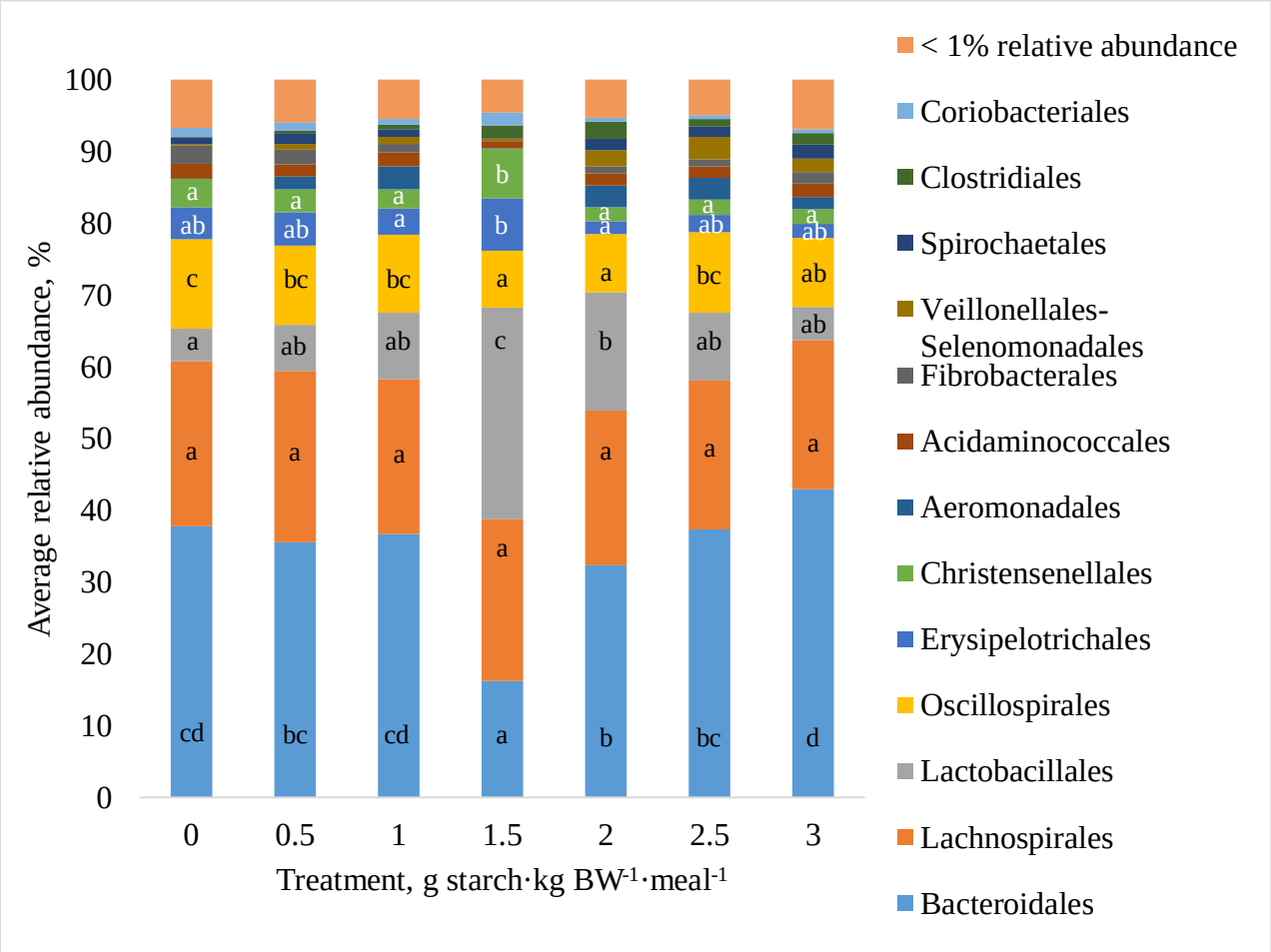
[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[a.b.c.d,e]Means within each taxa with a different letter differ, $P \leq 0.05$.

Figure 6.2. Average relative abundance of bacterial order in equine cecal digesta[1] when horses were fed increasing levels of dietary starch[*].



[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[a.b.c.d,e]Means within each taxa with a different letter differ, $P \leq 0.05$.

Figure 6.3. Average relative abundance of bacterial orders in equine cecal digesta[1] when horses were fed increasing levels of dietary starch[*].

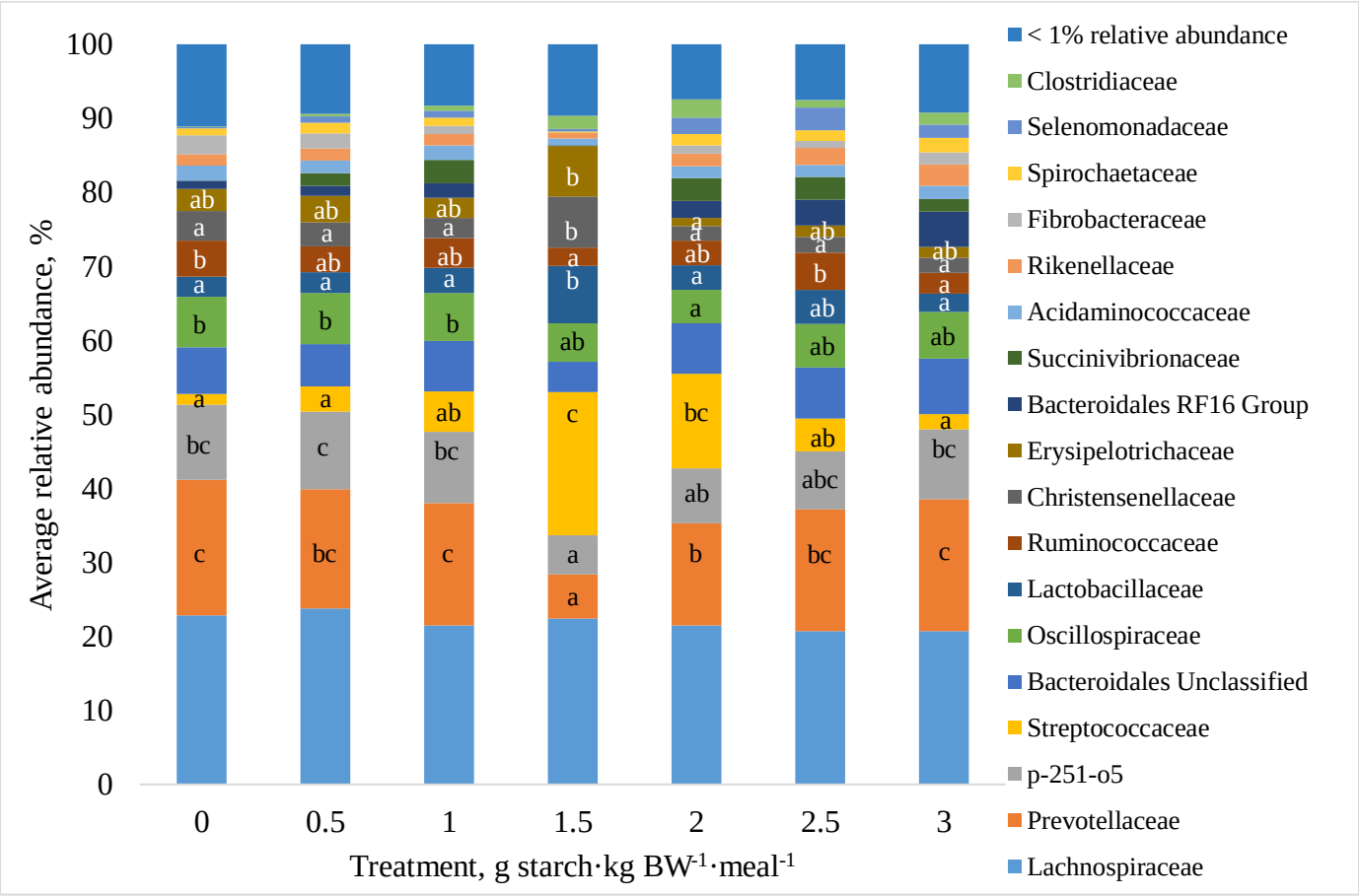


[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

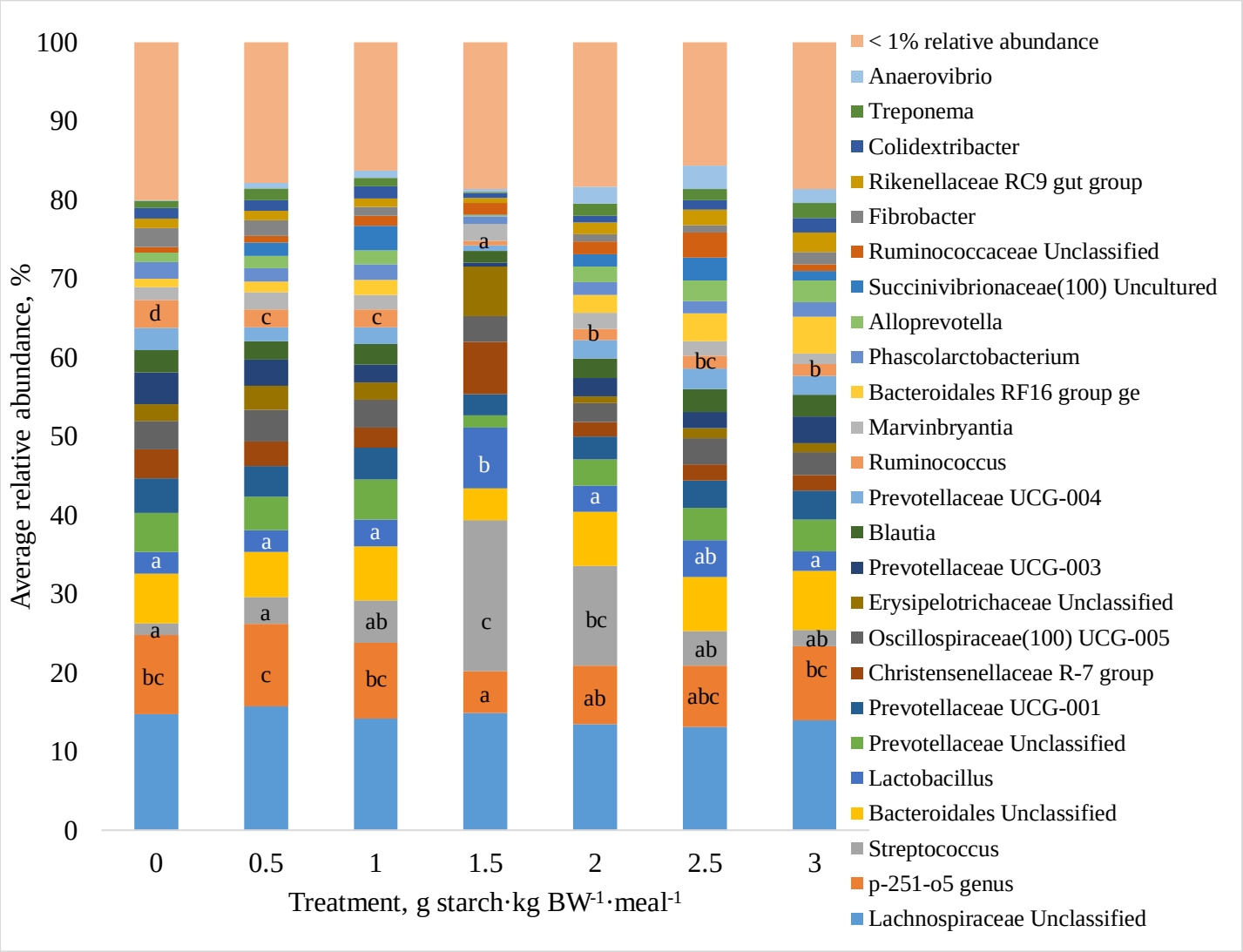
[a.b.c.d]Means within each taxa with a different letter differ, $P \leq 0.05$.

Figure 6.4. Average relative abundance of bacterial families in equine cecal digesta[1] when horses were fed increasing levels of dietary starch[*].



[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[a.b.c]Means within each taxa with a different letter differ, $P \leq 0.05$.

Figure 6.5. Average relative abundance of bacterial genera in equine cecal digesta[1] when horses were fed increasing levels of dietary starch[*].

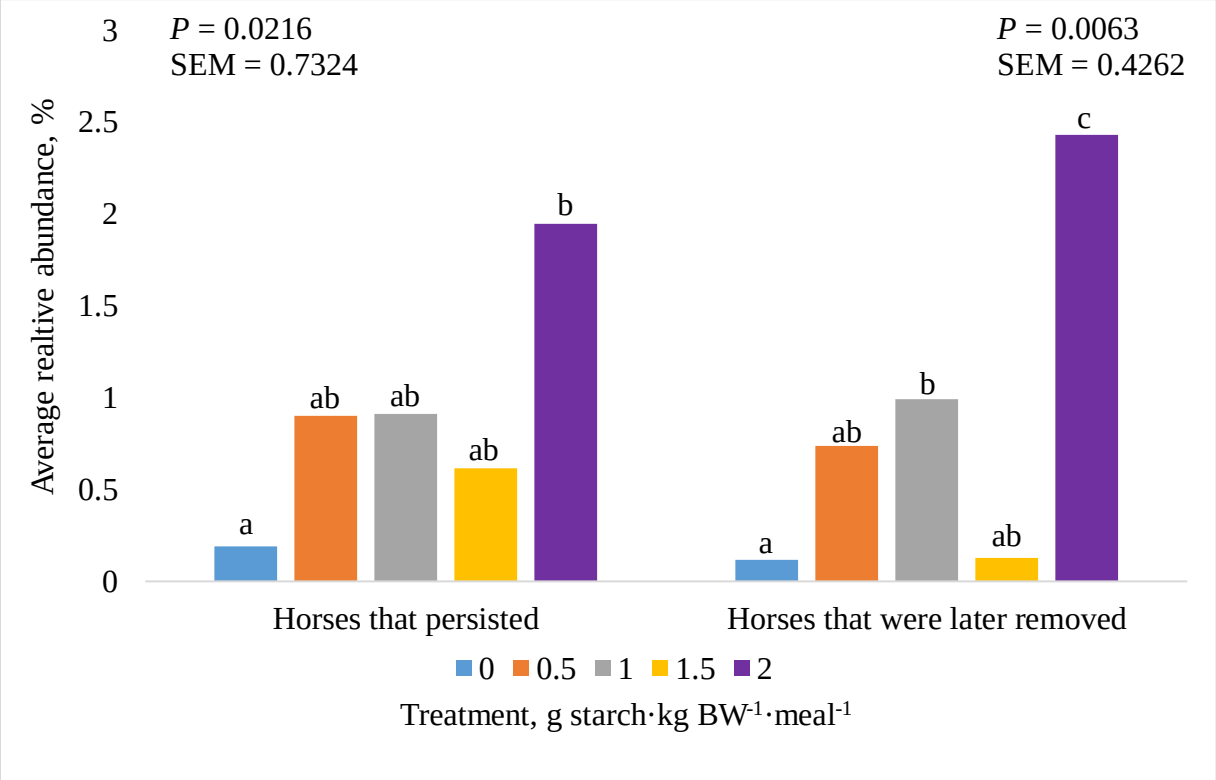


[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

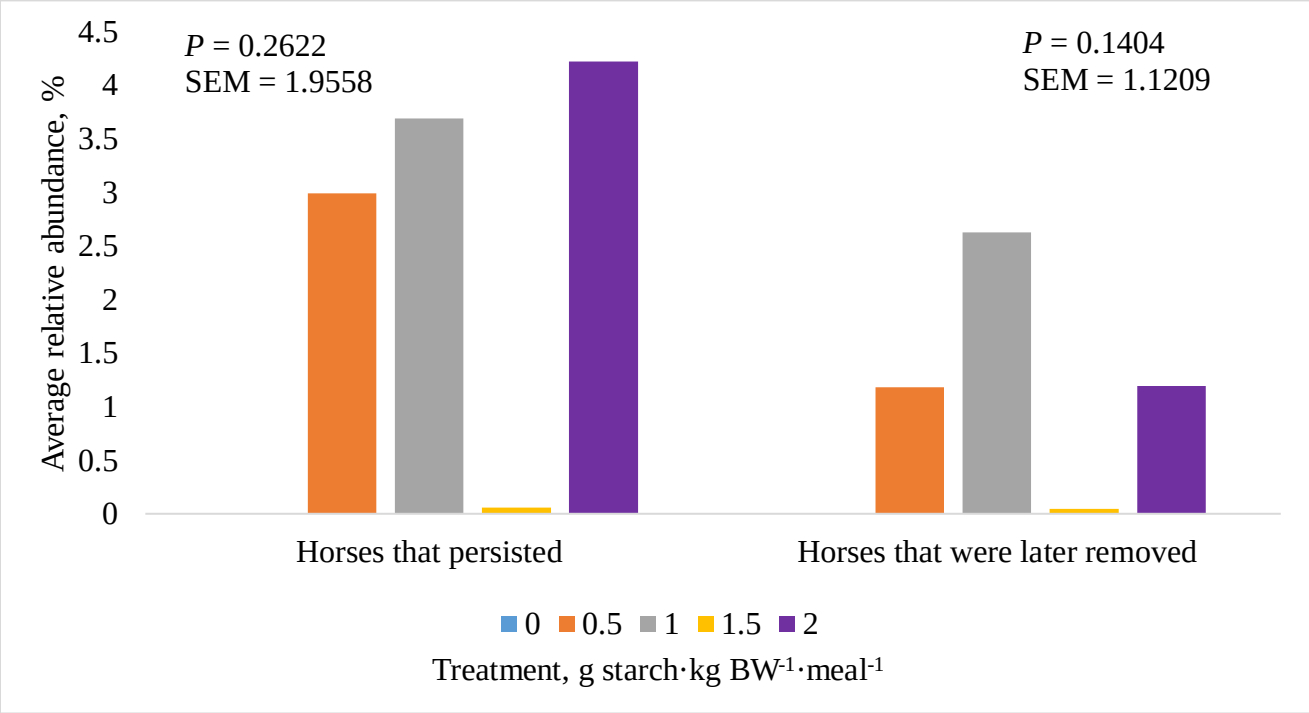
[a.b.c]Means within each taxa with a different letter differ, $P \leq 0.05$.

Figure 6.6. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Selenomondaceae* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



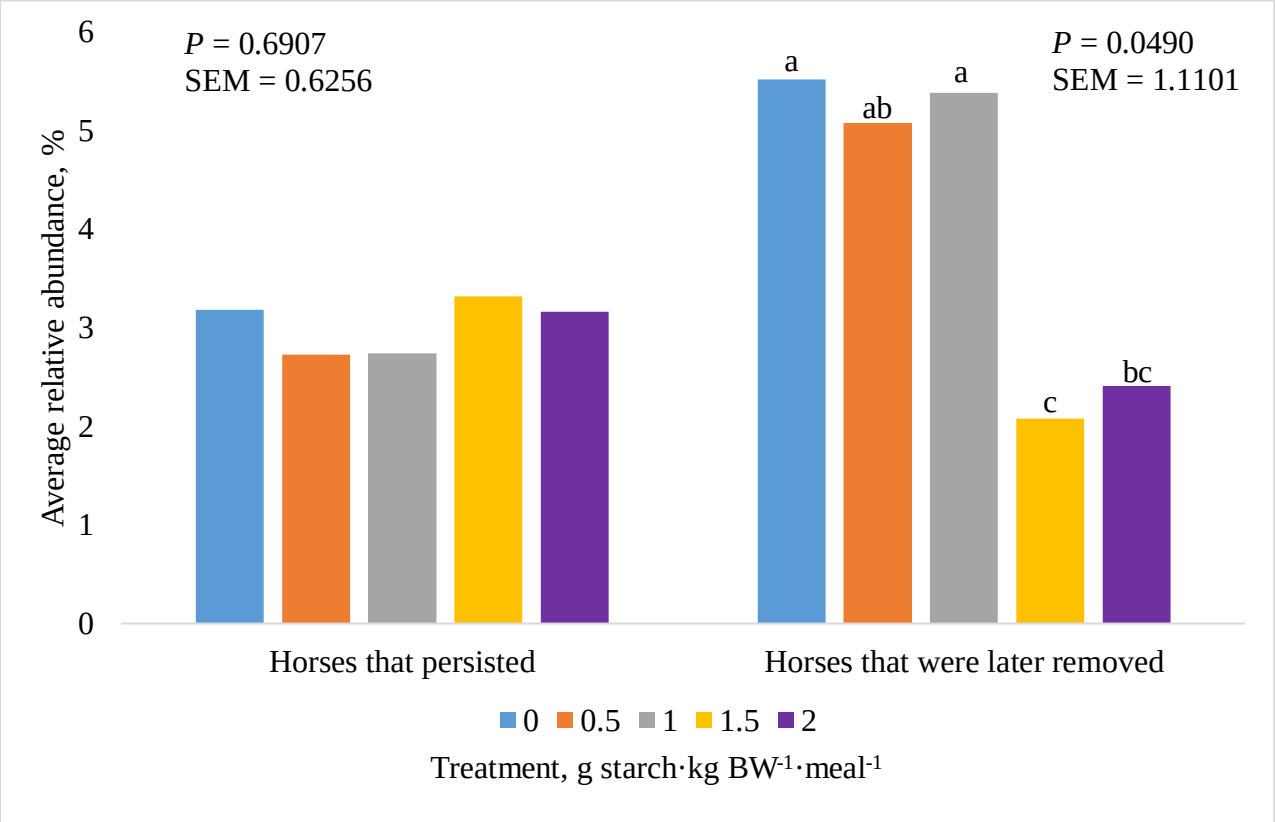
[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[a.b.c]Means within each group with a different letter differ, $P \leq 0.05$.

Figure 6.7. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Succinivibrionaceae* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



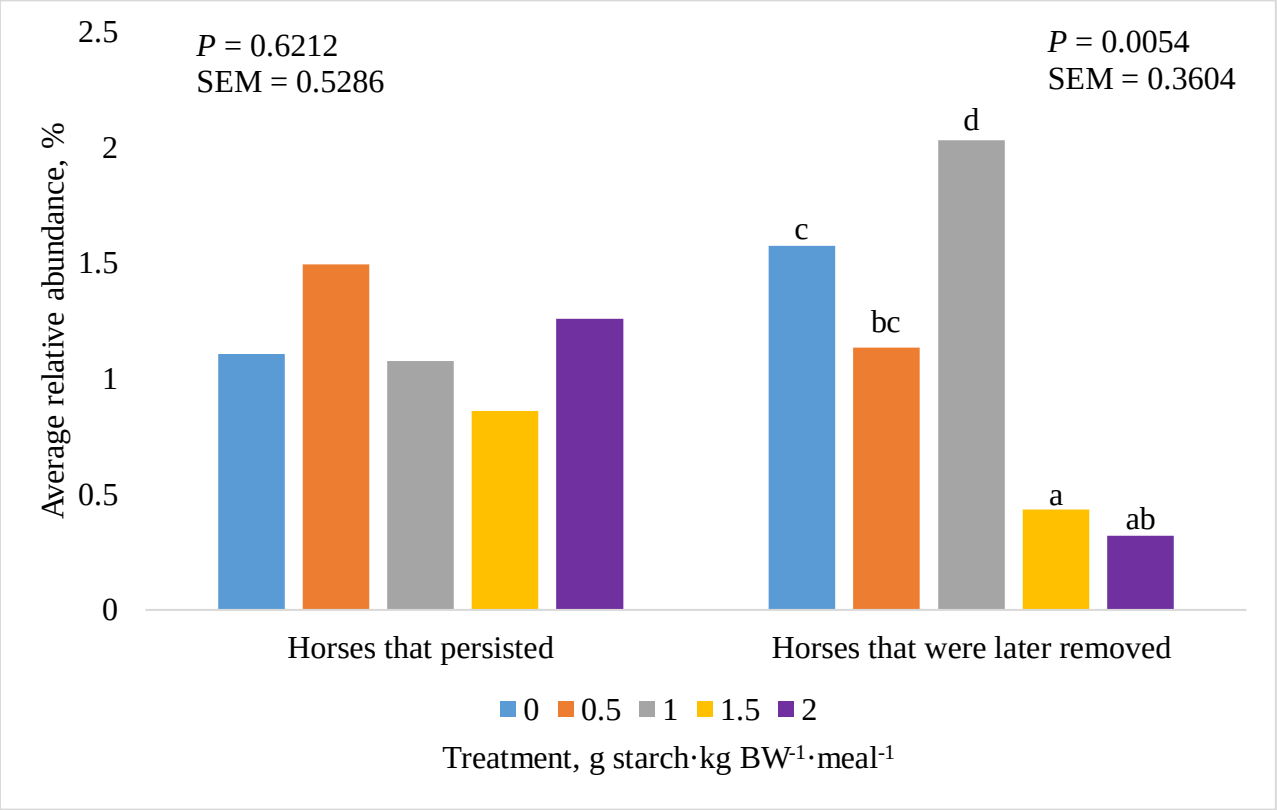
[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

Figure 6.8. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Prevotellaceae UCG-001* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.
[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.
[a.b.c]Means within each group with a different letter differ, $P \leq 0.05$.

Figure 6.9. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Colidextribacter* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.

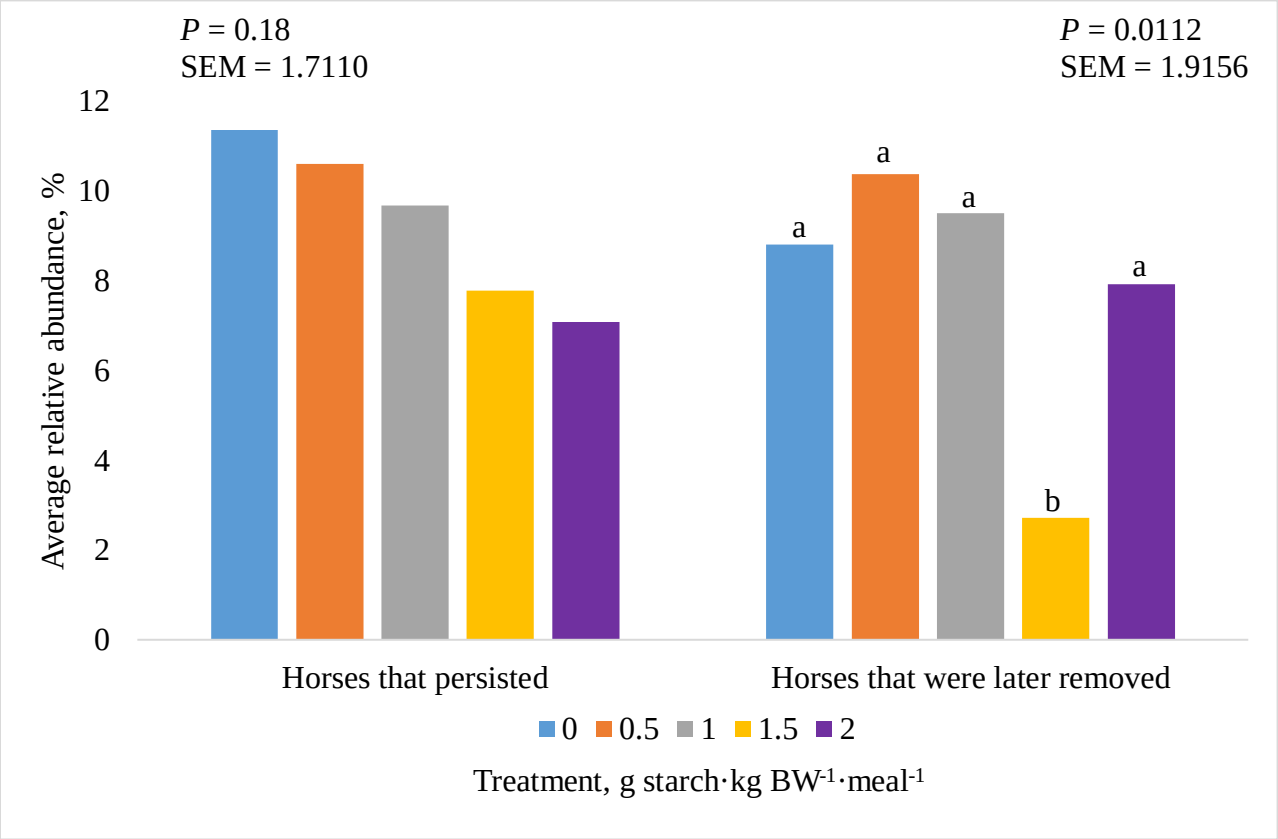


[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[a.b.c,d]Means within each group with a different letter differ, $P \leq 0.05$.

Figure 6.10. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *p-251-o5* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.

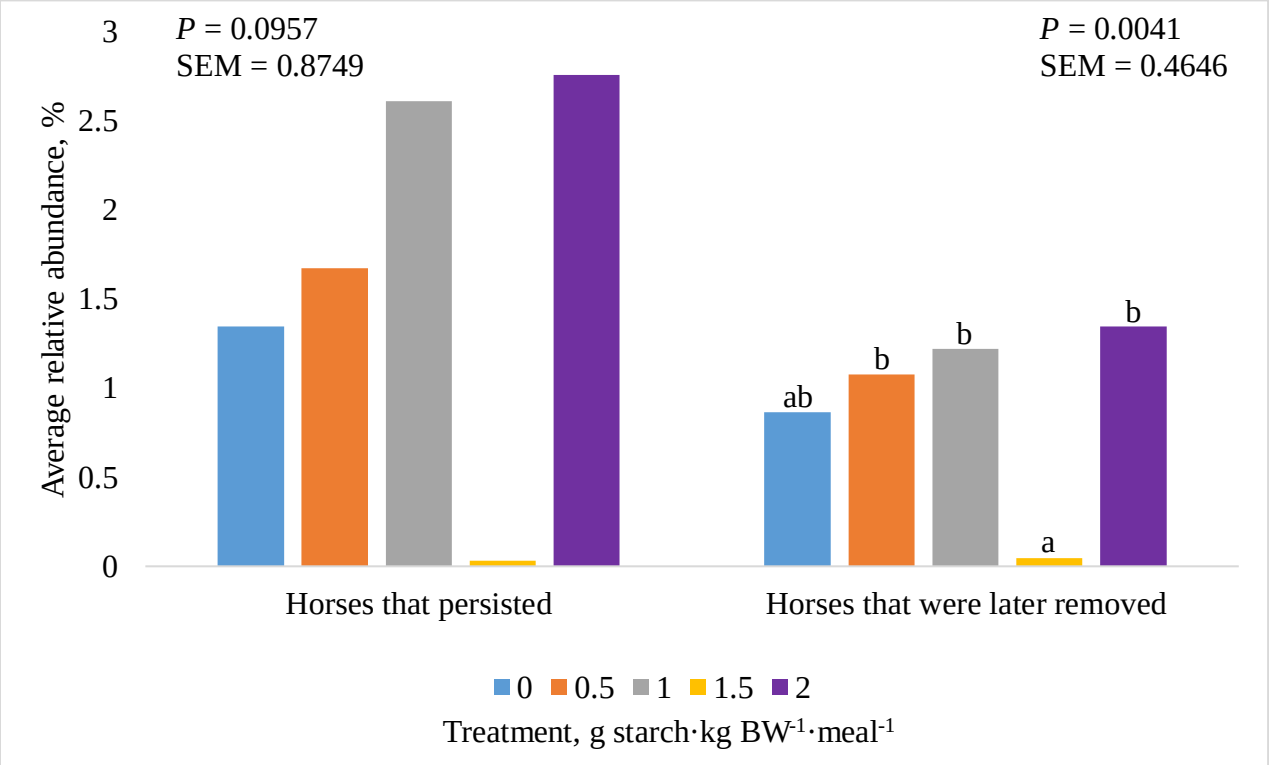


[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[a.b]Means within each group with a different letter differ, $P \leq 0.05$.

Figure 6.11. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *RF16 group* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.

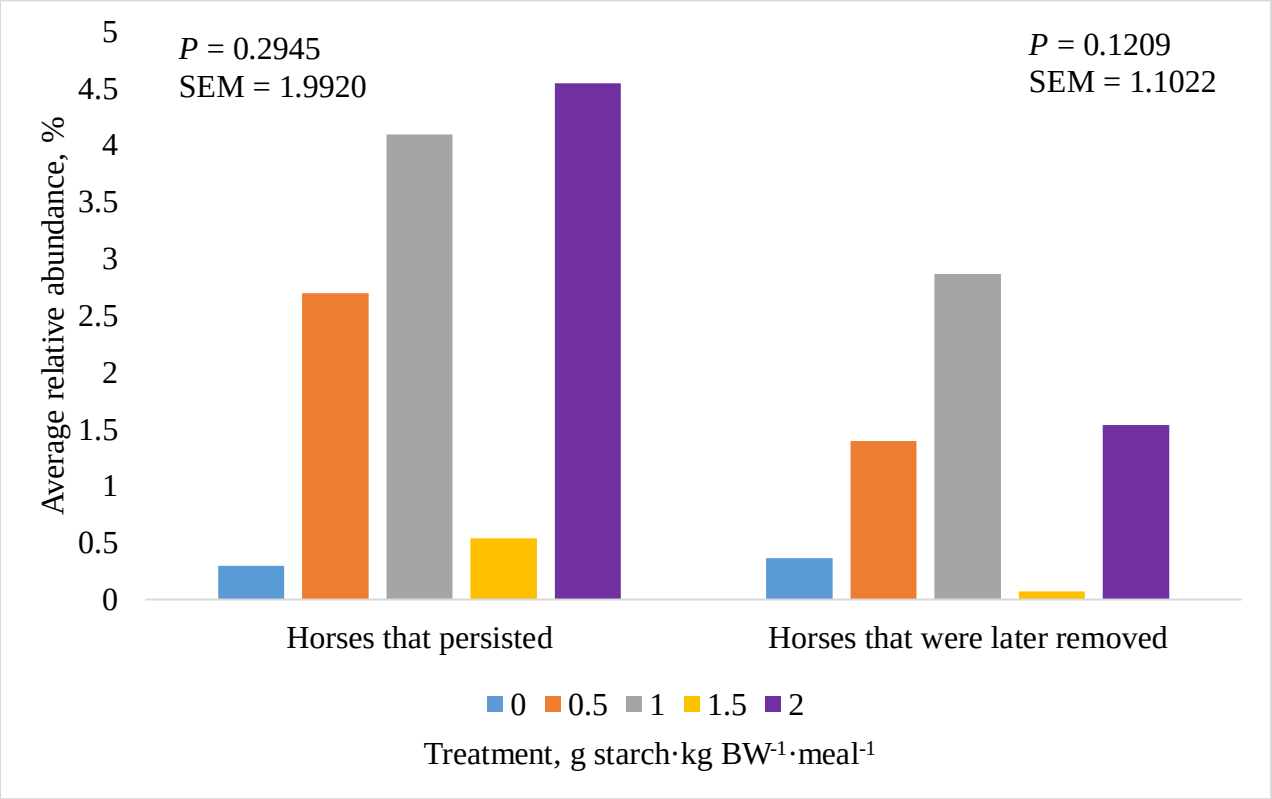


[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[a.b]Means within each group with a different letter differ, $P \leq 0.05$.

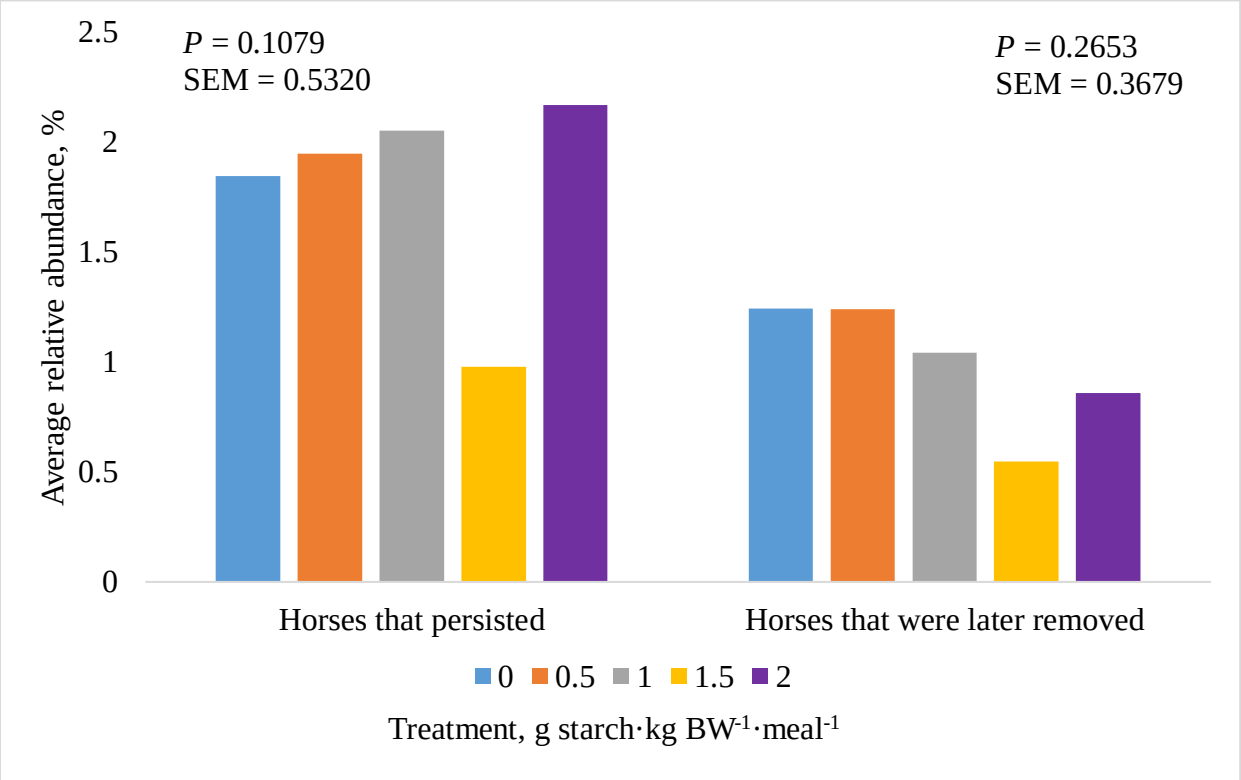
Figure 6.12. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Gammaproteobacteria* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

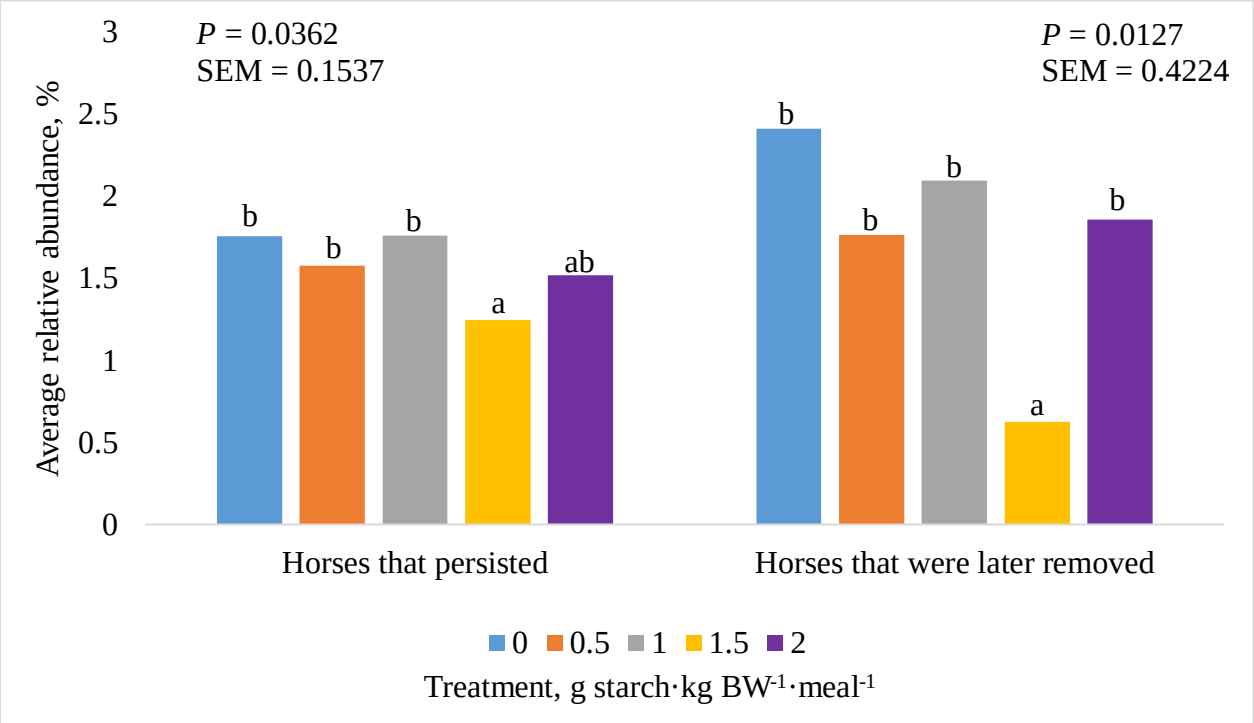
Figure 6.13. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Rikenellaceae* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

Figure 6.14. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Phascolarctobacterium* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.

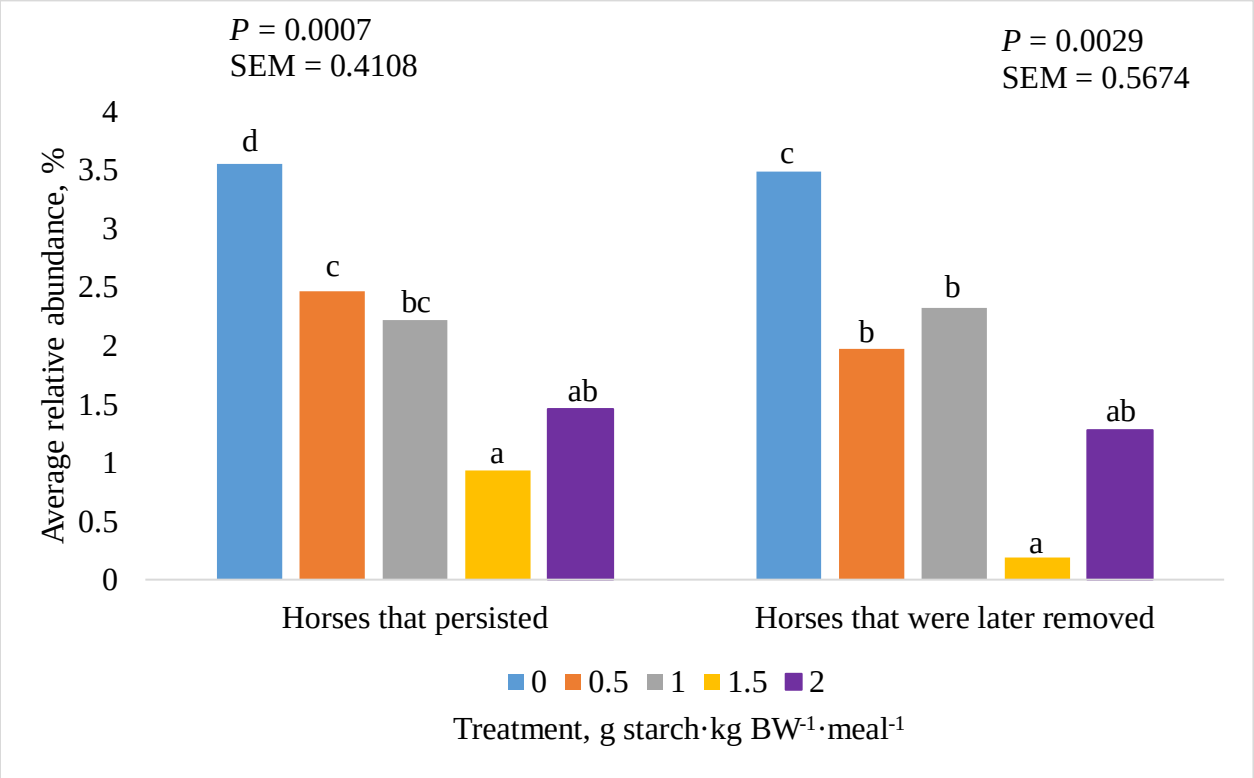


[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[a.b]Means within each group with a different letter differ, $P \leq 0.05$.

Figure 6.15. Effect of feeding increasing levels of dietary starch[*] on average relative abundance of *Ruminococcus* in equine cecal digesta[1] in horses that persisted and those removed from the experiment.



[*]Horses were offered *ad libitum* smooth bromegrass hay, 0.0125% BW/meal ration balancer, and increasing quantities of additional dietary starch (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g starch·kg BW^{-1}·$meal^{-1}$) supplied via pelleted corn and provided in 4 meals/d.

[1]Collected every 2 h for 12 h relative to the 0600 h feeding. Samples obtained from all time points at a given dietary starch level within an individual horse were pooled.

[a.b,c]Means within each group with a different letter differ, $P \leq 0.05$.

Chapter 7 - Effects of sodium caseinate and varying nitrogen sources on *in vitro* fermentation of forages by mixed equine cecal microorganisms

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Conflict of interest

None.

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ABSTRACT

Dietary protein recommendations for equines are not consistent and may not account for microbial nitrogen requirements in the equine hindgut. To assess the impact of nitrogen on fermentation by equine cecal microorganisms, cecal fluid from 4 cecally cannulated horses was used to inoculate fermentation bottles containing buffer, forage, and supplemental nitrogen. In experiment 1, sodium caseinate (SC) provided 0, 0.5, 1, 2, or 4% additional CP to bottles containing alfalfa (22.4% CP) or native warm season prairie grass hay (4.8% CP). Bottles were equipped with continuous gas pressure monitors and placed into a shaking incubator for 48 h at 39°C. Cultures with alfalfa had greater ($P < 0.0001$) *in vitro* dry matter disappearance (IVDMD), NDF disappearance (NDFD), ADF disappearance (ADFD), cumulative gas production and total VFA than those with grass hay. All levels of sodium caseinate increased gas production ($P \leq 0.05$) and decreased pH ($P < 0.003$) in cultures with grass hay. Sodium caseinate at 1, 2, or 4% additional CP increased IVDMD, NDFD, and ADFD ($P < 0.01$), while 4% additional CP also increased total VFA ($P < 0.01$) in cultures with grass hay. For experiment 2, SC, fishmeal, soybean meal (SBM), whey, porcine blood plasma, and L-lysine hydrochloride were added to supply 2% additional CP equivalent to cultures with grass hay. All nitrogen sources decreased pH and increased IVDMD, NDFD and ADFD ($P \leq 0.01$), with the largest effects elicited by SC, L-lysine, and whey ($P \leq 0.05$). Total VFA ($P \leq 0.04$) and gas ($P \leq 0.05$) production increased with L-lysine, whey, SC, SBM, and fishmeal. While nitrogen supplementation had minimal effects on cultures containing alfalfa, it altered fermentation and increased digestibility, as measured by IVDMD, NDFD, and ADFD, of grass hay, more notably with more soluble protein sources.

Key words*:* cecum; horse; in vitro; fiber; nitrogen

1 Introduction

In forage-fed ruminants, rumen degradable protein is often the first-limiting nutrient for microbial fermentation of feedstuffs [1]. Although ruminal conditions and protein structure are relevant, protein solubility often is used to predict ruminal degradability [2-4]. Ruminally degraded protein is converted to ammonia and utilized by microorganisms for proliferation, cellular maintenance and repair, and metabolic functions [4-6]. When availability of N is limited, as is often the case with low-quality roughages, the number and activity of microorganisms decreases, thus impeding degradation of fiber and subsequent volatile fatty acid (VFA) production [5,7]. Consequently, energy availability to the animal decreases as VFA's are the primary energy source for cattle [8].

Like the rumen, the cecum of the horse contains a large population of microorganisms that degrades fibrous feedstuffs [9,10,11,12]. Microbial populations within the equine hindgut may also experience limited N availability when horses consume low-protein roughages [11-14]. In fact, this is more likely in the horse, as protein is digested and absorbed in the small intestine, thereby reducing the amount of dietary N that appears in the hindgut [9,11,12,14,15]. There is increased VFA production, microbial proliferation, and disappearance of neutral detergent fiber (NDFD) and acid detergent fiber (ADFD) both in vivo and in vitro when additional N is provided to cecal microorganisms [12,14,16,17]. Degradation of protein within the cecum of horses increases availability of growth factors, such as branched-chain fatty acids (BCFA), to microorganisms. This results in greater microbial proliferation which leads to increased fermentation of substrates and subsequent VFA production. With more complete utilization of feedstuffs and increased energy available to both microorganisms and the horse, there is

increased feed efficiency which leads to decreased feed costs, which is of particular concern in times of drought when forage supply and quality are low.

While in vitro fermentation cannot duplicate feed utilization as it would occur in vivo, it does provide valuable information regarding how efficiently microorganisms ferment and use available nutrients. Previous researchers have illustrated the importance of protein availability for microorganisms within the equine hindgut; however, data are inconclusive concerning level of dietary N required and the impact of nitrogen source. Thus, the purpose of the following experiments was to evaluate effects of supplemental nitrogen on in vitro fermentation of alfalfa or native warm season prairie grass hay in batch cultures of mixed equine cecal microorganisms.

2. Materials and Methods

2.1 Animals

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. Four American Quarter Horses previously fitted with cecal cannulae (flexible rumen cannula, #7c; 3.8 cm center diameter and 8.9 cm wall thickness: Bar Diamond, Parma, ID; [18]) served as donors of cecal digesta. Horses were group housed in a dry lot (14.63 m x 34.14 m) with *ad libitum* access to water, bromegrass hay, and salt. For experiment 1, horses were group fed once per day at an approximate rate of 0.5% body weight (BW; as-fed) of a pelleted corn and oat-based concentrate that was mixed at the O. H. Kruse Feed Technology Innovation Center at Kansas State University (Table 7.1). For experiment 2, horses were again group fed once per day at an approximate rate of 0.5% BW of a commercially available pelleted concentrate (Strategy, Purina Mills Inc., St. Louis, MO). Horses had been maintained on respective diets ≥ 2 wk prior to collection of cecal digesta.

2.2 Experiment 1

2.2.1 Experimental Design

Experiment 1 was designed to evaluate effects of varying quantities of additional protein supplied via sodium caseinate, a highly soluble protein source [1,2], on fiber degradation of alfalfa (ALF) or native warm season prairie grass hay (GH) by mixed equine cecal microorganisms. This experiment was a randomized complete block design with a 2 x 5 (substrate x sodium caseinate inclusion rate) factorial arrangement of treatments. There were 4 blocks (horse) per treatment with fermentation bottle as the experimental unit. Substrate consisted of ALF or GH (Table 7.2). Sodium caseinate was added to provide 0 (control), 0.5, 1, 2, or 4% of additional crude protein (CP) on a dry matter (DM) basis. These rates were based on data collected previously in our laboratory [19].

2.2.2 In Vitro Fermentation

Substrate was ground to pass through a 1-mm Wiley mill screen (Model 4, Thomas Scientific, Philadelphia, PA). Five grams (DM) of ALF or GH, sodium caseinate, and cellulose were placed in 250-mL fermentation bottles (#7056; Ankom Technology, Macedon, NY). Cellulose was included at levels inversely proportional to sodium caseinate to equalize DM content between all bottles. Bottle contents were combined with 140 mL McDougall's buffer [20] and placed in a 39°C incubator for 4 h prior to collection of cecal digesta. Approximately 1 L cecal contents were obtained via gravity flow from each donor horse and strained through 4 layers of cheesecloth into pre-warmed, insulated containers. To minimize oxygen exposure, insulated containers were completely filled with cecal digesta. Cecal fluid was immediately

transported to the laboratory where it was strained through another 4 layers of cheesecloth, placed into separatory funnels, sparged with N_2 to create an anaerobic environment, capped with a rubber stopper, and placed in a 39°C incubator (Isotemp 550D Incubator Oven; Fisher Scientific, Hampton, NH). After 30 min, cecal fluid had stratified into 3 layers (sediment, floating mat, and intermediate fluid). Ten milliliters of intermediate fluid were added to each fermentation bottle as microbial inoculum. Additionally, for each block (horse) there was a blank that contained only buffer and intermediate fluid. Blanks were used to determine baseline gas production and VFA concentrations.

Immediately following microbial inoculation, initial pH was measured using a portable pH meter (Thermo Scientific Orion 3 Star Portable pH Meter, Waltham, MA). Bottles were gassed with N_2, fitted with Ankom gas monitoring modules (RF Gas Production System; Ankom Technology Corp., Macedon, NY), and placed in a 39°C shaking incubator (G25; New Brunswick Scientific Inc., New Brunswick, NJ) for 48 h. Cumulative gas production was recorded every 15 min for 48 h, at which point cultures were exposed to oxygen and terminal pH was recorded. One module containing GH and 0.5% additional CP malfunctioned; consequently, gas production data from this module were not included in the statistical analysis.

2.2.3 Analyses of VFA

Upon termination, 4 mL liquid were removed from each bottle, mixed with 1 mL 25% (wt/vol) metaphosphoric acid, and frozen at -20°C. Two milliliters of thawed, vortexed (Scientific Industries Vortex-Genie 2, Houston, TX), and deproteinized cultures were later placed into microcentrifuge tubes and centrifuged at 17,000 × g for 15 min. Then 1.5 mL supernatant were transferred to gas chromatography vials (#958896, Cobert Associates, St.

Louis, MO), vortexed, and placed onto an Agilient 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a Nukol capillary column (15 m length, 530 μm diameter, 0.5 μm film thickness; Supelco columns; Sigma-Aldrich, St. Louis, MO) and flame ionization detector. Hydrogen gas served as the carrier with a flow rate through the column of 4.5126 mL/min. Inlet temperature was set at 275°C and oven temperature originated at 100°C and was increased by 10°C/min until a final oven temperature of 220°C was reached. Detector temperature was 300°C. Total run time per sample was 14 min. Volatile fatty acid concentrations were quantified by comparing individual VFAs to that of a known standard (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO).

2.2.4 Analyses of DM, Neutral Detergent Fiber (NDF), and Acid Detergent Fiber (ADF)

Remaining contents of culture bottles were transferred into pre-weighed aluminum tins and placed in a forced air oven at 55°C for 5 d. Percent in vitro dry matter disappearance (IVDMD) was determined using the equation $\frac{\mathrm{Ws}-[(\mathrm{Wds}-\mathrm{Wp})-(\mathrm{Wc}-\mathrm{Wp})]}{\mathrm{Ws}} \times 100$ where Ws represents the weight of substrate weighed into fermentation bottles (DM basis), Wds represents the weight of dried sample and pan, Wp denotes pan weight and Wc represents dry weight of the corresponding blank sample and pan.

Residual dry sample was ground manually using a ceramic mortar and pestle and 0.5 g was transferred into fiber filter bags (Ankom Technologies; SKU 57). Neutral detergent fiber and ADF were determined in samples, GH, and ALF using the method described by Goering and Van Soest (Ankom Technology Corp., Fairport, NY) [21]. Results from these analyses were used to determine percent NDFD or ADFD by using the equation Ws - $\frac{Wf-(Wds-Wb)}{Wf} \times 100$ where

Ws is g of NDF or ADF present in substrate, Wf represents g weighed into fiber bags (DM basis), Wds represents g NDF or ADF in the dry sample plus bag weight, and Wb represents bag weight in g.

2.2.5 Statistical Analyses

Data obtained from blank cultures were not included in the statistical analyses but were subtracted from corresponding culture bottles containing treatments. Blank-corrected values were used in statistical analyses. Data were analyzed using the MIXED procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC). Fermentation bottle served as the experimental unit. For gas production, the model included random effect of block (horse) and fixed effects of concentration of sodium caseinate, forage type, time, and all 2- and 3-way interactions. Repeated measures were utilized to evaluate effects over time. Models for pH, VFA, IVDMD, NDFD and ADFD included fixed effects of concentration of sodium caseinate, forage type, and their interaction with random effect of block (horse). Least-squares means (LSMEANS) were calculated using the PDIFF option. Differences between LSMEANS were considered significant at $P < 0.05$ and tendencies were declared at $0.05 < P < 0.10$.

2.3 Experiment 2

2.3.1 Experimental Design

A second experiment was conducted to evaluate effects of varying nitrogen sources on fiber degradation of GH in batch cultures of mixed cecal microorganisms. This experiment was a randomized complete block design with 7 treatments consisting of a negative control (cellulose) and 6 nitrogen sources [sodium caseinate, whey, L-lysine hydrochloride (L-lysine), soybean

meal, fishmeal, and porcine blood plasma (plasma); Table 7.3]. All nitrogen sources were added at levels calculated to supply 2.0% additional CP (DM) equivalent, as 2% additional nitrogen produced measurable effects on response variables measured in Experiment 1. There were 4 blocks (horse) per treatment with fermentation bottle as the experimental unit.

2.3.2 In Vitro Fermentation

Utilizing the same laboratory procedures as described in section 2.2.2, culture bottles were inoculated with cecal fluid, buffer, 5 g (DM) GH, and the appropriate nitrogen source. Each block contained a culture bottle without added nitrogen that served as control. Cellulose was added at rates calculated to equalize DM between all culture bottles. Within each block, a blank containing only strained cecal fluid and buffer was included to determine baseline gas pressure and VFA concentrations. Gas production and terminal pH were monitored as described in section 2.2.2.

2.3.3 Analyses of VFA

Volatile fatty acids were analyzed using the procedure described in section 2.2.3 with the following adjustments. Hydrogen served as the carrier gas with a flow rate through the column of 4.7492 mL/min. Inlet temperature was 300°C and oven temperature initially was set at 70°C and increased 15°C/min to reach a final temperature of 200°C. Total run time per sample was 14.6 min.

2.3.4 Analyses of DM, NDF, and ADF

In vitro dry matter disappearance, NDFD and ADFD were analyzed in the same manner described in section 2.2.4.

2.3.5 Statistical Analyses

Values obtained from blank cultures were subtracted from those obtained in corresponding treatment cultures to generate blank-corrected values that were included in the statistical analyses. Data were analyzed using the MIXED procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC). Experimental unit was fermentation bottle. For gas production, the model included nitrogen source, time, and their interaction as fixed effects and block (horse) as a random effect. Repeated measures were used to evaluate the effect of nitrogen source over time. The models for pH, VFA, IVDMD, NDFD, and ADFD included the fixed effect of nitrogen source and random effect of block (horse). Least-squares means were calculated using the PDIFF option. Significance was declared at $P < 0.05$ and a tendency at $0.05 < P < 0.10$.

3. Results and Discussion

3.1 Experiment 1

There were forage × sodium caseinate interactions on gas production ($P < 0.0001$; Fig. 7.1), pH, IVDMD, NDFD, and ADFD ($P \leq 0.0008$; Table 7.4) whereby sodium caseinate had no effect in cultures containing ALF ($P > 0.05$). With 22.4% CP, ALF likely supplied sufficient N to meet microbial requirements for proliferation and repair. Compared to GH, ALF also contained more soluble CHO, which are more rapidly fermented by microorganisms than structural CHO. Simultaneous availability of N and CHO results in increased microbial proliferation and fermentation of feedstuffs [10,12,13,22]. Accordingly, there was increased gas production from h 11 to 48, total VFA, IVDMD, and ADFD and decreased pH in cultures

312 containing ALF compared to those containing GH, regardless of sodium caseinate
313 supplementation ($P \leq 0.0339$).
314 Cultures containing GH (4.8% CP) benefited from supplementation of sodium caseinate,
315 likely because GH did not provide adequate CP to meet N requirements for microorganisms.
316 Greater inclusion rates of sodium caseinate led to more rapid increases in gas pressure, which
317 was used as an indicator of microbial activity as CO_2, CH_4, H_2S, and other gasses are released
318 during fermentation [23,24]. Cultures containing 2 and 4% supplemental CP had increased ($P \leq$
319 0.0498) gas production from h 23 to 48 compared to GH control, from h 28 to 48 compared to
320 cultures with 0.5% added CP ($P \leq 0.0477$), and from h 32 to 48 compared to cultures containing
321 1% added CP ($P \leq 0.0407$). Cultures containing 1% supplemental CP had increased ($P \leq 0.0476$)
322 gas production from h 30 to 48 compared to GH control. All cultures containing supplemental
323 CP and GH had increased ($P \leq 0.0495$) gas production compared to GH control from h 44 to 48.
324 Santos et al. [14] also reported increased gas production when protein was added to N-deficient
325 cultures of equine cecal microorganisms. Cultures containing GH with supplemental CP had
326 decreased ($P \leq 0.0225$) pH compared to GH control, which provides further evidence of
327 increased fermentative activity. While decreased pH is generally associated with increased
328 accumulation of organic acids, increased VFA concentrations were only observed with 4%
329 supplemental CP; however, intermediates, such as succinic and formic acids, were not analyzed
330 and may have affected pH.
331 Inclusion of supplemental CP did not affect total VFA concentration in cultures containing
332 ALF ($P > 0.05$). Four percent additional CP, however, led to increased concentrations of
333 branched-chain fatty acids (BCFA; isobutyrate, isovalerate and isocaproate) compared to ALF
334 control ($P \leq 0.0412$). Branched-chain fatty acids are direct products of fermentation of branched-

chain amino acids (BCAA) [4,25,26,27]. Li et al. [26] reported sodium caseinate contained 4.91, 8.82, and 6.03% of the BCAA isoleucine, leucine, and valine, respectively. Thus, supplementation with large amounts of sodium caseinate was expected to result in increased concentrations of BCFA. There were no differences in BCFA concentrations between ALF control cultures and those supplemented with 0.5, 1, and 2% CP ($P > 0.05$).

Total VFA were greater in cultures containing GH and 4% additional CP compared to GH control ($P \leq 0.0051$) and there was a tendency toward increased total VFA compared to cultures containing 0.5% added CP ($P = 0.0686$). Cultures supplied 1 and 2% additional CP had a tendency for greater total VFA compared to GH control ($P \leq 0.0853$). Inclusion of 4% additional CP increased acetate concentration compared to GH control ($P = 0.0109$) and cultures containing 2% additional CP tended to have greater acetate compared to GH control ($P = 0.08$). Acetate is the primary VFA produced from fermentation of fiber [24,28,29] and, as expected, was the VFA present in the greatest concentrations. Butyrate concentration was increased with 4% supplemental CP compared to GH control ($P = 0.0492$). Butyrate has been associated with maintaining proper gut integrity and thus improving health of the animal [30]. Cultures with 1, 2, and 4% additional CP had increased propionate compared to GH control ($P \leq 0.0491$). Accordingly, all inclusion rates of sodium caseinate with GH led to decreased acetate:propionate ratio (A:P) compared to GH control ($P \leq 0.0494$). Supplying soluble sodium caseinate to cecal microorganisms likely resulted in proliferation of proteolytic bacteria which tend to be more amylolytic than cellulolytic [26,31,32]. Compared to cellulolytic bacteria, amylolytic and proteolytic bacteria produce less acetate and greater proportions of propionate and its precursors [28]. Amylolytic bacteria decarboxylate succinate, a common end-product of fiber fermentation

by predominant fibrolytic bacteria, to propionate, thus reducing total proportion of acetate relative to propionate [24,28,29].

The inclusion of sodium caseinate to supply 2 and 4% additional CP in cultures containing GH resulted in greater isovalerate concentrations ($P \leq 0.0422$), while only the inclusion of 4% added CP led to increased isobutyrate ($P = 0.0002$) compared to GH control. Isocaproate was unaffected by supplemental CP ($P = 0.2699$) in cultures containing GH. This was somewhat unexpected as sodium caseinate increased production of all BCFA evaluated in cultures containing ALF.

There was a linear forage × sodium caseinate effect on IVDMD ($P < 0.0001$) whereby sodium caseinate inclusion had no effect on cultures containing ALF ($P > 0.05$); however, inclusion of ≥ 1% additional CP increased IVDMD compared to 0.5% added CP and control cultures ($P < 0.01$) provided with GH. Increasing sodium caseinate led to greater IVDMD in GH cultures, with 4% additional CP resulting in greater IVDMD compared to cultures containing 1% additional CP ($P = 0.04$). There were linear and quadratic forage × sodium caseinate interactions on NDFD ($P < 0.0001$, $P = 0.0085$, respectively) and ADFD ($P = 0.0005$, $P = 0.0032$, respectively). The inclusion of ≥ 1% added CP to cultures containing GH resulted in greater NDFD and ADFD compared to GH control and GH cultures containing 0.5% additional CP ($P \leq 0.001$). Cultures supplemented with 2 and 4% additional CP had the greatest NDFD ($P < 0.03$) of GH, thus it appears these levels of supplementation enhanced the ability of microorganisms in GH cultures to ferment hemicellulose, cellulose, or both [21].

Providing 4% additional CP to cultures containing GH did not increase IVDMD, NDFD, ADFD, production of gas and total VFA, nor decrease pH when compared to GH cultures with 2% added CP. At this level, nitrogen supplementation > 2% provided no further benefit. Because

the GH contained 4.8% CP, cultures with 2 and 4% added CP had total CP of 6.8 and 8.8%, respectively. From these data, it appears that supplying 6.8% CP to equine cecal microbiota meets its N requirement. A similar CP content of 7% is recommended for forage-fed cattle to avoid decreased nutrient digestibility [8,33]. Currently, the amount of dietary CP necessary to optimize microbial fermentation within the horse is not clearly defined, largely due to differences in the rate of pre-cecal digestion of protein in differing feedstuffs; however, it has been estimated that 11.4% (2.2 g/kg BW) nitrogen should be offered to horses to maximize NDF digestibility [16]. Conversely, the 2007 NRC recommends protein intake of 6.3% CP (1.26 g/kg BW) for horses in maintenance [34], which illustrates the conflicting recommendations concerning protein requirements in the horse. The ALF used in this experiment contained 22.4% CP, greatly surpassing the recommended 7% CP for cattle supplied low-quality forages [8,33] as well as the 6.3 and 11.4% CP recommended for horses by the 2007 NRC [34] and Oliveira et al. [16], respectively, thus rendering supplemental CP unnecessary. Jordan et al. [19] confirmed that an optimal protein requirement is likely within this range as they observed no effect on fermentation parameters (cecal pH, cecal VFA, and feed digestibility) nor feed intake when sodium caseinate was dosed directly into the equine cecum when horses were fed hay containing 8.5% CP. Pre-cecal digestion of protein, however, is influenced by rate of passage and feed ingredient [11,15] thus, the optimal levels of dietary CP required to maximize microbial fermentation will also be affected by these parameters.

In the present experiment, substrate disappearance was limited, especially in cultures containing GH. This was likely impacted by considerable NDF and ADF content in the substrate (73.2% and 51.8%, respectively), which would reduce rate and extent of fermentation [35]. The 19 h lag period in gas production for cultures containing GH (Fig. 7.1) provides evidence that the

403 rate of fermentation was indeed slow in these cultures. Greater IVDMD would likely have been
404 observed if fermentation had been allowed to continue for a longer period of time, as gas
405 production curves had not yet reached a plateau when the experiment ceased. Furthermore, only
406 20% of ruminal microorganisms can be cultured [36] and, due to similarities between the equine
407 cecum and the bovine rumen, it is reasonable to assume that not all cecal microorganisms
408 remained viable after being transferred into culture.
409
410 **3.2 Experiment 2**
411 There was a nitrogen source x time interaction on gas production ($P < 0.0001$; Fig. 7.2). In
412 experiment 2, gas accumulated at a faster rate in cultures containing more soluble nitrogen. Gas
413 production was greater with sodium caseinate, whey, and L-lysine than control by h 27 ($P \leq$
414 0.029). Cumulative gas production was greater ($P \leq 0.0154$) with the inclusion of all nitrogen
415 sources, except plasma, compared to control by h 44. There was a tendency for cultures
416 containing plasma to have increased gas production over the control at h 48 ($P = 0.09$). Sodium
417 caseinate supplementation resulted in the greatest cumulative gas production ($P \leq 0.0013$), which
418 cannot be entirely explained by nitrogen solubility, as whey and L-lysine both contain protein
419 that is 100% soluble.
420 Regardless of source, supplemental nitrogen led to decreased terminal pH ($P < 0.0001$; Table
421 7.5) compared to control, with the greatest depressions occurring with sodium caseinate, whey,
422 and L-lysine ($P \leq 0.0185$). Organic acid concentration is the driving factor behind pH and thus, it
423 was expected cultures with lower pH would have greater VFA concentrations. Total VFA
424 concentrations were increased ($P \leq 0.0401$) with the inclusion of all nitrogen sources, except
425 plasma, compared to control (Table 7.5). Cultures containing plasma had a tendency to have

greater total VFA concentration than control ($P = 0.0564$). Increased total VFA were driven, at least in part, by increases in acetate ($P \leq 0.0286$) and butyrate ($P \leq 0.0373$) in cultures containing sodium caseinate, whey, and L-lysine compared to control. All nitrogen sources resulted in increased propionate ($P \leq 0.0215$) and a corresponding decrease in the A:P ratio ($P \leq 0.0005$) compared to control. Valerate concentrations were increased in cultures containing fishmeal, plasma, sodium caseinate, and whey ($P \leq 0.0245$) compared to control.

Cultures containing plasma, sodium caseinate, and whey had greater isovalerate ($P \leq 0.0172$) and isobutyrate ($P \leq 0.0104$) compared to control. This likely reflects increased concentrations of BCAA in these nitrogen sources as it has been reported milk proteins, such as sodium caseinate and whey, have greater BCFA concentrations, followed by other animal-based nitrogen sources, than plant-based nitrogen sources [4,26,27]. Valerate and BCFA are required by several cellulolytic bacteria as growth factors [24,28]. Thus, supplying cecal microbiota with nitrogen sources containing BCAA is expected to stimulate growth of cellulolytic bacteria. In the present study, while increases in BCFA were observed with plasma, whey, and sodium caseinate, this does not appear to be the driving factor behind degradation of substrate as all supplemental nitrogen sources led to increased ADFD ($P < 0.0001$), NDFD ($P < 0.0001$), and IVDMD ($P \leq 0.0138$) compared to control. Sodium caseinate, whey, and L-lysine elicited the greatest NDFD ($P \leq 0.0385$) and ADFD ($P \leq 0.0370$). Sodium caseinate, whey, L-lysine and plasma elicited the greatest ($P \leq 0.0305$) IVDMD.

Across response variables, sodium caseinate, whey, and L-lysine consistently elicited larger responses, presumably due to increased nitrogen solubility. Solubility, however, does not always align with degradability of nitrogen. Plasma, which contained 83.0% soluble CP, increased substrate disappearance compared to control but did not result in increased gas or VFA

449 production in the present study. Plasma contains many disulfide bonds, which microorganisms
450 are less efficient at hydrolyzing [2], thus resulting in a gradual release of ammonia and amino
451 acids. This results in more gradual production of organic acids compared to other highly soluble
452 nitrogen sources. Consequently, growth of microorganisms is expected to be slower, thus
453 cumulative gas and total VFA production were expected be less in cultures containing plasma as
454 compared to cultures supplemented with other highly soluble nitrogen sources. The greater
455 sodium content of plasma also is known to impact fermentation [37]. Cattle maintain consistent
456 rumen osmolarity with sodium-rich diets by increasing water consumption [38]; however,
457 diffusion of water to maintain osmolarity cannot occur in a fermentation bottle. In the present
458 study, plasma contained 2.32% sodium (DM basis) whereas all other nitrogen sources contained
459 ≤ 1.37% sodium (DM basis). As a result, ionic balance may have been altered as plasma was
460 degraded, negatively impacting fermentation. There is, however, evidence that some bacterial
461 species require greater sodium concentrations than those normally found in the rumen [24], so
462 the effect of sodium would be largely dependent on the makeup of the bacterial population
463 present. Meanwhile, soybean meal, which contains approximately 20% soluble CP, is considered
464 85% rumen degradable and is rapidly fermented within the rumen [39]. The discrepancy between
465 rumen degradability and solubility of soybean meal is due to the fractionalization of CP
466 [39,40,41,42]. In the current experiment, solubility of CP of soybean meal was more indicative
467 of the effects of soybean meal on fermentation parameters.
468

469 ## 5. Conclusions

470 It is apparent from experiment 1 that equine cecal microorganisms increase fermentative
471 activity when supplemental CP is provided with a low-quality warm season prairie grass hay

containing 4.8% CP. There was no additional benefit gained in substrate disappearance if > 2% additional CP was provided. Conversely, additional CP had no impact on fermentation parameters when alfalfa with 22.4% CP was provided as the substrate. Thus, it was apparent that alfalfa alone supplied adequate N to support maximal fermentative activity.

In experiment 2, all nitrogen sources impacted microbial fermentation of poor-quality grass hay, but those sources with greater solubility (sodium caseinate, whey, and L-lysine) led to the greatest increase in microbial activity, as measured by gas and VFA production, pH, and substrate disappearance. Translating these findings to the live animal may prove challenging, as much dietary nitrogen is digested and absorbed in the small intestine, making it difficult to provide nitrogen supplementation to the microbiota of the equine hindgut. Regardless, it is clear that nitrogen amount and source impact fermentation of forages by equine cecal microorganisms.

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588

Table 7.1. Nutrient composition (dry matter basis) of concentrates* fed to horses used as donors of cecal digesta.

Component, %	corn and oat-based[1]	Strategy[2]
Dry matter (DM)	86.27	--
Crude protein	15.40	14.00
Crude fat	5.40	6.00
Acid detergent fiber	9.00	15.50
Neutral detergent fiber	8.00	34.00
Calcium	1.04	0.90-1.40
Phosphorous	0.78	0.60
Magnesium	0.17	--
Potassium	0.97	--
Digestible energy, Mcal/kg, DM	3.73	--

*Fed at a rate of 0.5% body weight (as-fed) once per day

[1]Proximate analysis of custom mix prepared at Kansas State University; fed during experiment 1

[2]Values obtained from feed tag (Purina Mills Inc., St. Louis, MO); fed during experiment 2

Table 7.2. Proximate analysis of alfalfa and native warm season prairie grass hay (dry matter basis) used as substrate for *in vitro* fermentation by mixed equine cecal microorganisms*.

Item	Alfalfa hay	Grass hay
Dry matter, %	90.4	92.9
Neutral detergent fiber, %	39.7	73.2
Acid detergent fiber, %	31.4	51.8
Crude protein (CP), %	22.4	4.8
Soluble protein, % CP⸸	38.0	28.0
Degradable protein, % CP	78.0	43.0
Ether extract, %	2.2	7.4
Non-structural carbohydrates, %	6.2	3.4
Water soluble carbohydrates, %	5.3	2.2
Ethanol soluble carbohydrates, %	2.9	2.1
Ash, %	10.2	7.4
Digestible energy, Mcal/kg	2.4	1.8

*Proximate analyses using wet chemistry (Dairy One Forage Lab, Ithaca, NY)
⸸Analyzed via the Cornell Sodium Borate-Sodium Phosphate Buffer Procedure (Dairy One Forage Lab, Ithaca, NY)

Table 7.3. Proximate analysis of nitrogen sources (dry matter basis)*† supplied to mixed cecal microorganisms.

Item	Soybean meal	Fishmeal	Plasma	Sodium caseinate	Whey	L-lysine
Dry matter, %	90.800	92.800	87.400	91.800	94.60	95.300
Crude protein (CP), %	52.700	65.400	84.800	92.200	77.90	93.900
Soluble protein, % CP⸸	22.000	24.000	83.000	95.000	100.00	100.000
Sodium, %	0.048‡	1.367	2.319	0.723	0.21	< 0.001

*Soybean meal, fishmeal, porcine blood plasma (plasma), sodium caseinate, whey and L-lysine hydrochloride (L-lysine) supplied 2% additional CP equivalent to mixed equine cecal microorganisms provided with 5 g (dry matter basis) of native warm season prairie grass hay
†Proximate analyses using wet chemistry (Dairy One Forage Lab, Ithaca, NY)
⸸Analyzed via the Cornell Sodium Borate-Sodium Phosphate Buffer Procedure (Dairy One Forage Lab, Ithaca, NY)
‡Reference value reflects the mean Na content in 2,544 samples collected from 2000 to 2018 (Dairy One Forage Lab, Interactive Forage Library, Ithaca, NY)

Table 7.4. Effect of sodium caseinate[1] on terminal pH and volatile fatty acid concentrations (VFA), *in vitro* dry matter disappearance (IVDMD), neutral detergent fiber disappearance (NDFD), and acid detergent fiber disappearance (ADFD) in batch cultures of cecal microorganisms.

	Grass hay					Alfalfa hay						*P*-value		
% added CP[2]	0	0.5	1	2	4	0	0.5	1	2	4	SEM	Forage	SC[3]	F × SC[4]
Terminal pH	6.57^{a}	6.39^{b}	6.34^{bc}	6.12^{d}	6.21^{cd}	5.80^{e}	5.75^{e}	5.85^{e}	5.78^{e}	5.85^{e}	0.0744	< 0.0001	0.0025*‡	0.0008*‡
Acetate, mM	23.13^{a}	35.54^{ab}	42.55^{ab}	44.10^{ab}	54.66^{b}	122.54^{c}	104.54^{c}	120.27^{c}	116.10^{c}	126.44^{c}	11.5414	< 0.0001	0.1319*	0.3527
Propionate, mM	9.92^{a}	17.60^{ab}	21.58^{b}	23.05^{b}	28.27^{b}	47.56^{c}	41.54^{c}	45.03^{c}	48.42^{c}	44.47^{c}	5.6576	< 0.0001	0.2259*	0.1435
A:P[5]	2.31^{a}	2.01^{b}	1.99^{b}	1.97^{b}	1.94^{b}	2.58^{acd}	2.56^{acd}	2.70^{cd}	2.48^{ac}	2.86^{d}	0.1428	< 0.0001	0.2187	0.0466*
Isobutyrate, mM	0.0^{a}	0.35^{a}	0.59^{a}	0.70^{a}	1.69^{b}	2.40^{bcd}	2.00^{bc}	2.50^{cd}	2.90^{de}	3.54^{e}	0.3880	< 0.0001	< 0.0001*	0.6736
Butyrate, mM	4.24^{a}	6.88^{ab}	7.91^{ab}	7.47^{ab}	10.23^{b}	22.49^{cd}	18.22^{c}	22.30^{cd}	22.44^{cd}	25.04^{d}	2.9100	< 0.0001	0.1581*	0.5910
Isovalerate, mM	0.00^{a}	0.42^{ab}	0.90^{ab}	0.94^{b}	2.44^{c}	2.83^{cd}	2.32^{c}	2.99^{cd}	3.57^{de}	4.35^{e}	0.4425	< 0.0001	< 0.0001*	0.5144
Valerate, mM	0.00^{a}	0.27^{a}	0.75^{a}	1.09^{ab}	2.58^{bc}	2.83^{bde}	2.73^{bd}	3.86^{cde}	4.56^{e}	4.53^{e}	0.8577	< 0.0001	0.0068*	0.7603
Isocaproate, mM	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.45^{ab}	1.01^{bc}	0.80^{ac}	1.07^{acd}	1.43^{cd}	1.87^{d}	0.3995	< 0.0001	0.0839*	0.7294
Caproate, mM	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.26^{ab}	0.55^{ab}	0.93^{bc}	1.24^{d}	1.10^{dc}	0.2814	< 0.0001	0.2774	0.2774
Total VFA	37.28^{a}	61.07^{ab}	74.26^{ab}	77.37^{ab}	100.33^{b}	202.09^{c}	172.63^{c}	198.97^{c}	200.66^{c}	211.34^{c}	20.6990	< 0.0001	0.0795*	0.3618
IVDMD[7], %	11.07^{a}	10.68^{a}	15.05^{b}	17.45^{bc}	18.13^{c}	33.73^{d}	34.79^{d}	33.59^{d}	34.59^{d}	32.10^{d}	1.3959	< 0.0001	0.0042*‡	0.0001*
NDFD[8], %	31.62^{a}	31.92^{a}	40.31^{b}	44.87^{c}	44.73^{c}	48.04^{cd}	48.91^{d}	51.25^{d}	50.30^{d}	50.81^{d}	1.9293	< 0.0001	< 0.0001*‡	0.0002*‡
ADFD[9], %	27.31^{a}	30.26^{a}	38.39^{b}	42.75^{b}	42.22^{b}	51.52^{c}	50.91^{c}	53.48^{c}	52.58^{c}	54.62^{c}	2.1960	< 0.0001	< 0.0001*‡	0.0004*‡

[1]Sodium caseinate included to supply 0, 0.5, 1, 2, or 4% additional protein to mixed equine cecal microorganisms used as inoculum in 48-h fermentation cultures containing 5 g (DM basis) alfalfa (22.4% CP) or warm season native prairie grass hay (4.8% CP)
[2]Crude protein
[3]Main effect of sodium caseinate
[4]Forage × sodium caseinate interaction
[5]Acetate:propionate ratio
[*]Linear effect ($P < 0.05$)
[‡]Quadratic effect ($P < 0.05$)
[a,b,c,d,e]Means within a row without a common superscript differ ($P < 0.05$)

Table 7.5. Effect of nitrogen source* on terminal pH, volatile fatty acid concentration (VFA), *in vitro* dry matter disappearance (IVDMD), neutral detergent fiber disappearance (NDFD), and acid detergent fiber disappearance (ADFD) for batch cultures containing mixed cecal microorganisms.

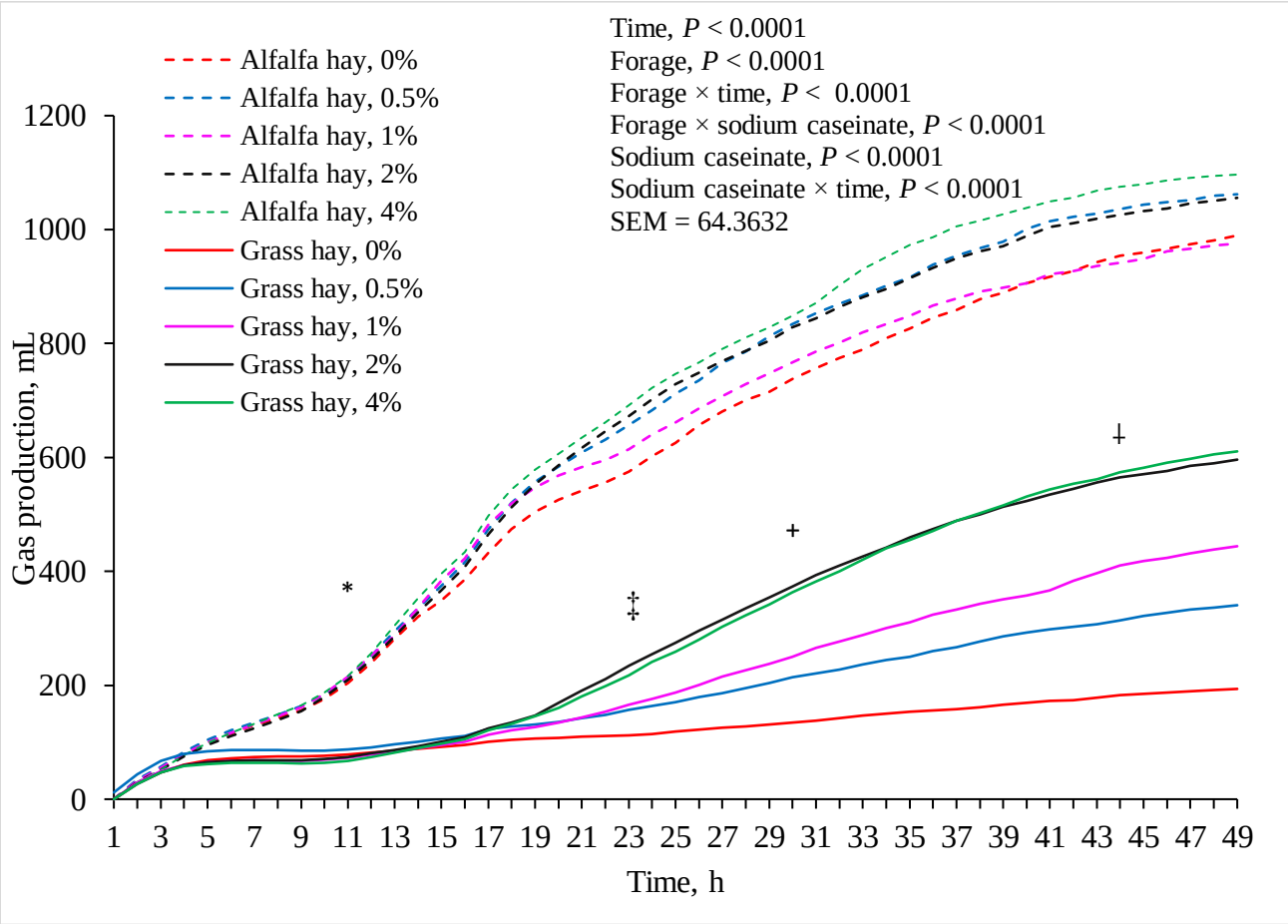
Item	Nitrogen source							SEM	*P*-value
	Control	Soybean meal	Fishmeal	Plasma	Sodium caseinate	Whey	L-lysine		Nitrogen
Terminal pH	6.76^{a}	6.53^{b}	6.51^{bc}	6.46^{c}	6.33^{d}	6.33^{d}	6.28^{d}	0.0261	< 0.0001
Acetate, mM	13.68^{a}	26.82^{ab}	26.70^{ab}	25.21^{ab}	33.54^{b}	32.44^{b}	28.70^{b}	6.3134	0.0992
Propionate, mM	4.58^{a}	11.01^{b}	11.13^{b}	10.97^{b}	14.81^{b}	15.08^{b}	12.73^{b}	2.5391	0.0124
A:P[1]	2.95^{a}	2.37^{b}	2.39^{b}	2.20^{b}	2.21^{b}	2.11^{b}	2.17^{b}	0.1337	< 0.0001
Isobutyrate, mM	0.12^{a}	0.23^{ab}	0.25^{ab}	0.31^{bc}	0.44^{c}	0.43^{c}	0.17^{ab}	0.0645	0.0006
Butyrate, mM	1.49^{a}	3.05^{ab}	2.95^{ab}	2.80^{ab}	3.27^{bc}	3.75^{bc}	4.73^{c}	0.7908	0.0289
Isovalerate, mM	0.13^{a}	0.28^{ab}	0.29^{ab}	0.35^{b}	0.57^{c}	0.64^{c}	0.15^{a}	0.0857	< 0.0001
Valerate, mM	0.08^{a}	0.21^{ab}	0.24^{b}	0.23^{bc}	0.47^{d}	0.35^{cd}	0.19^{ab}	0.0598	0.0002
Isocaproate, mM	0.00^{a}	0.01^{a}	0.01^{a}	0.02^{a}	0.01^{a}	0.03^{a}	0.01^{a}	0.0117	0.2969
Caproate, mM	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.01^{a}	0.0067	0.4552
Total VFA	20.08^{a}	41.60^{b}	41.57^{b}	39.89^{ab}	53.10^{b}	52.72^{b}	46.70^{b}	9.7127	0.0518
IVDMD, %	8.80^{a}	12.09^{b}	12.47^{b}	15.07^{c}	15.35^{c}	15.31^{c}	15.98^{c}	1.1980	0.0001
NDFD, %	31.64^{a}	40.72^{b}	40.40^{b}	42.18^{b}	44.93^{c}	45.48^{c}	44.87^{c}	1.1991	< 0.0001
ADFD, %	25.76^{a}	35.66^{b}	36.16^{b}	36.78^{b}	40.33^{c}	40.41^{c}	41.54^{c}	1.6992	< 0.0001

*Soybean meal, fishmeal, porcine blood plasma (plasma), sodium caseinate, whey, and L-lysine hydrochloride (L-lysine) were included to supply 2% additional protein equivalent to 48-h fermentation cultures containing 5 g (DM basis) of native warm season prairie grass hay and inoculated with mixed equine cecal microorganisms

[1]Acetate:propionate ratio

[a,b,c]Within a row, means without a common superscript are different ($P < 0.05$)

Figure 7.1. Effect of sodium caseinate† on gas production by mixed equine cecal microorganisms.



†Sodium caseinate included to supply 0, 0.5, 1, 2, or 4% additional protein to mixed equine cecal microorganisms used as inoculum in 48-h fermentation cultures containing 5 g (DM basis) alfalfa or warm season native prairie grass hay

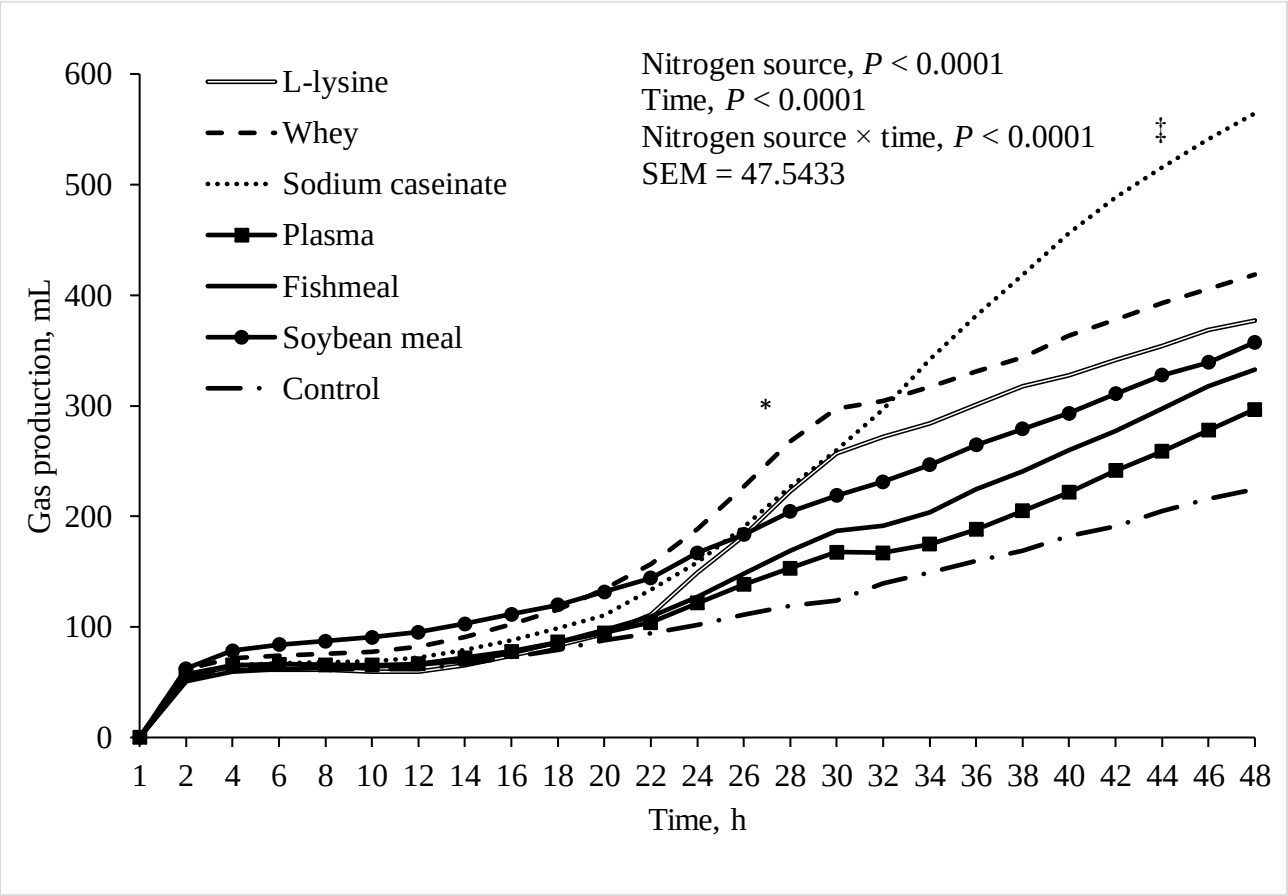
*Cultures containing alfalfa had increased gas production from h 11 to 48 compared to cultures containing native warm season prairie grass hay ($P \leq 0.0339$)

‡Cultures containing native warm season prairie grass hay with 2 or 4% additional protein had increased gas production from h 23 to 48 compared to cultures containing native warm season prairie grass hay and no additional protein ($P \leq 0.0498$)

+Cultures containing native warm season prairie grass hay with 1% additional protein had increased gas production from h 30 to 48 compared to cultures containing native warm season prairie grass hay and no additional protein ($P \leq 0.0476$)

ǂCultures containing native warm season prairie grass hay with 0.5% additional protein had increased gas production from h 44 to 48 compared to cultures containing native warm season prairie grass hay and no additional protein ($P \leq 0.0495$)

Figure 7.2. Effect of nitrogen source† on gas production by mixed equine cecal microorganisms



†Sodium caseinate, L-lysine hydrochloride (L-lysine), porcine blood plasma (plasma), soybean meal, whey or fishmeal was included to supply 2% additional protein equivalent to mixed equine cecal microorganisms in 48-h fermentation cultures containing 5 g (DM basis) warm season native prairie grass hay

*From h 27 to 48 gas production was greater in cultures containing sodium caseinate, whey and L-lysine compared to control ($P \leq 0.0372$)

‡From h 44 to 48 cultures with sodium caseinate, L-lysine, soybean meal, whey and fishmeal had greater gas production than control ($P \leq 0.0422$)