Effect of sodium caseinate on hindgut fermentation and fiber digestion in the equine and Effect of *Megasphaera elsdenii* on broiler chick performance

by

Katherine Van Jordan

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Approved by:

Major Professor
James M. Lattimer

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Abstract

Eight cecally cannulated horses were used in a replicated, 4 x 4 Latin square design conducted in 4, 14-d periods to determine effects of sodium caseinate on equine hindgut fermentation and fiber digestion. Horses were maintained on an *ad libitum* diet of Smooth Bromegrass hay and treatments consisted of cecal infusions of water (CON), 0.125 g sodium caseinate/kg BW (LOW), 0.25 g sodium caseinate/kg BW (MED), and 0.50 g sodium caseinate/kg BW (HI). Cecal NH_3 concentrations increased ($P < 0.01$) as casein increased. Horses on the CON and MED treatments had greater cecal pH ($P < 0.01$) than horses on the LOW and HI treatments; however, there was no effect of treatment of fecal pH. Dry matter, OM, NDF, and ADF digestibility were unaffected by treatment ($P > 0.10$). There were no differences in dry matter intake (DMI), regardless of treatment. There was no treatment effect on cecal acetate, propionate, butyrate, total VFA concentration or acetate:propionate (A:P) ratio. Inter-horse variability and a small sample size may contribute to the lack of statistical differences amongst treatments. Results from this study may imply that medium quality, roughage based protein supplies an adequate level of nitrogen to the microflora of the hindgut.

A performance study was conducted to evaluate the effect of strain and method of administration of *Megasphaera elsdenii* on growth performance in broiler chickens. In Experiment 1, pens were randomly assigned to 1 of 6 treatments: 0.2 mL oral gavage containing 1.97 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 (Lactipro, MS-Biotec, Wamego, KS; O-L), 0.2 mL of fresh culture containing 0 CFU/mL of *M. elsdenii* strain KS 249 (O-KS; Attempts to grow this strain were unsuccessful), 0.2 mL of a fresh culture containing 1.06 x 10^9 CFU/mL of *M. elsdenii* strain B52-2083 (O-B52), aerosolized mist at rate of 15 mL per pen containing 1.97 x109 CFU/mL of *M. elsdenii* strain NCIMB 41125 (~1.88 mL/bird; MS-Biotec, Wamego,

KS; OM), topdressing (mixture of diet and freeze dried *M. elsdenii* strain NCIMB 41125; TD) containing 1.18 x 10^7 CFU/g of *M. elsdenii* strain NCIMB 41125 at a rate of a quarter teaspoon per bird, or negative control that had no contact with the probiotic product. Broilers across all treatment groups showed similar ADFI ($P = 0.82$), ADG ($P = 0.89$), gain:feed ($P = 0.93$), and mortality rates ($P = 0.54$). In Experiment 2, chicks were assigned to 1 of 2 treatments consisting of lyophilized *M. elsdenii* strain NCIMB 41125 in the form of a topdressing (TD) or a negative control that had no contact with the probiotic product (C). Average daily gain ($P = 0.02$) and gain:feed ($P = 0.04$) were both greater in birds receiving the TD when compared to the C birds. Feed intake ($P = 0.70$) and mortality rates ($P = 0.31$) were not different between treatments. Administration of lyophilized *M. elsdenii* strain NCIMB 41125 may be an effective means to improve growth rate and feed efficiency of broiler chicks.

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Dedication

To my parents, Bill and Michelle Jordan. Thank you for supporting my horse habit throughout the years.

Chapter 1 - Literature Review:

Protein and Fiber Digestion in the Horse

Introduction

Equine diets typically are comprised of both forages and concentrates, which can vary in their proportions. Due to performance demands many horses consume diets rich in concentrates which may lead to consequences such as gastric ulcers and laminitis. On the other end of the spectrum, many horses have access to readily available native forages in the form of both hay and pasture. While this diet accommodates the natural feeding pattern of the equine, many native grasses can be of poor quality which leads to decreased fiber digestibility and nutrient utilization.

Microbial growth in the hindgut and subsequent fiber fermentation can be reduced by limited nitrogen availability, which may be the case with poor quality forages. This has been well documented in the ruminant, where supplemental nitrogen increases ruminal fermentation and digestibility of low-protein forages (Köster et al., 1996; Loest et al., 2000). This phenomenon is relatively unexplored in the horse. Given the similarities between the rumen and cecum, the same observation could be expected. This effect may be even more dramatic in the horse because a large proportion of available protein is digested and absorbed in the small intestine (Russell, 2007; Santos et al., 2012).

In this review, fiber and protein digestion in the gastrointestinal tract of the equine is examined. Moreover, it examines how increasing nitrogen availability to the microbial population of the cecum might improve microbial fermentation.

Protein Digestion

Because specific amino acids (AA) are required for protein synthesis, the horse's requirement is for AA rather than protein. To meet these requirements, protein from the diet is broken down into single AA before being absorbed. To date, only one individual AA requirement has been established that of lysine (NRC, 2007). Amino acids that cannot be synthesized in the body at levels necessary to meet the animal's demand for them are considered essential AA and must be provided in the diet. (NRC, 2007).

Protein digestion begins in the stomach with the secretion of hydrochloric acid (HCl) and pepsinogen. Hydrochloric acid denatures dietary protein, exposing peptide bonds to digestive enzymes. In addition, HCl activates pepsinogen to pepsin which hydrolyzes the peptide chain to produce polypeptides. The small intestine plays the primary role in protein digestion. Protein digestion in the small intestine involves several pancreatic enzymes and microvillar enzymes (Erickson and Kim, 1990). Digestion and absorption in the small intestine can account for 30 to 80% of total dietary protein digestion. (Geor et al., 2013).

Pancreatic enzymes tend to be present in larger quantities towards the proximal small intestine with microvillar enzymes being more prevalent in the ileum. Secretions from the pancreas include trypsinogen, chymotrypsinogen, and procarboxypeptidases A & B, all of which must be activated by trypsin. Trypsin is activated by enteropeptidase from the duodenal brush border membrane. Following processing by pancreatic peptidase activity, peptides are digested further by enterocyte enzymes in the brush border membrane. More prevalent in the jejunum and duodenum, these aminopetidases include dipeptidyl peptidase IV and carboxypeptidases. Di- and tri-peptides can be broken down further within the lumen, but many are absorbed as peptides and then cleaved within the enterocyte. Transport proteins are critical in facilitating the absorption of

AA from the small intestine to the bloodstream. In cattle, pigs, and chickens, it may be advantageous for the animal to absorb di-and tri-peptides as they are absorbed faster than individual AA and they represent over half of the AA absorbed . However, no peptide transporters have been identified in the small intestine of the horse (Geor et al., 2013). Only two studies have been conducted to examine the existence of specific AA transport proteins in the horse. Woodward et al. detected the $b^{0,+}$ system on the apical membrane and the y^{+} and L systems on the basolateral membrane of the small intestine (Woodward et al., 2010; Woodward et al., 2012).

After absorption into the bloodstream, AA are delivered to various tissues, such as muscle, for protein synthesis. Additionally, they can be further catabolized in the liver to produce glucose or be used as a precursor to keytone body production (Pelley, 2007). Excess AA and ammonia that are absorbed through the intestinal wall are carried to the liver through the portal-hepatic system. Most AA and ammonia extracted from the blood are converted to urea and excreted in feces and urine. Once excreted, nitrogen can be volatilized to ammonia. Ammonia can leach into surrounding soil and ground water, and it may create hazardous conditions for animals and workers breathing in contaminated air (Drummond et al., 1978; Olsman et al., 2003).

Fermentation of Nitrogen and Non-Protein Nitrogen in the Hindgut

Contradictory to the ruminant, little work has been done to examine the effect of protein on microbial fermentation in the equine. Since protein digestion primarily occurs in the small intestine, the quantity of protein reaching hindgut is unknown. Research conducted in the horse

to evaluate the large intestine's role in protein digestion has proven bacteria of the hindgut can utilize ammonia and urea as nitrogen sources.

Average NH_3 concentrations in the hindgut reportedly range from 3 to 10 mml (Hecker 1971; Schwabenbauer et al., 1982). Ammonia is readily transported across the mucosa of the large colon. In an *in vitro* setting study utilizing isolated equine large intestine mucosa, an average of 63% of the absorbed NH_3 was detected on the serosal side (Bochroder et al., 1994). Any NH^3 not absorbed will be excreted. In horses cecally dosed with N-labeled bacteria, AA were found in portal blood draining from the cecum thus indicating the horse is able to absorb AA and digest microbial protein within the large intestine (Slade et al., 1971). However, this was contradicted in a later studies. When studied in vitro, little to no AA were shown to cross through the apical membrane of either the cecum or the colon (Bochroder et al., 1994; Freeman et al., 1989; Freeman and Donawick, 1991; Wysocki and Baker, 1975). A later study identified only 2 apical membrane AA transporters in the large intestine (Woodward et al., 2012). These findings support the idea that AA can be absorbed in the large intestine of the horse, but only in small amounts.

In ruminants, protein can be classified into 2 categories to include ruminally degradable protein (RDP) and ruminally undegradable protein (RUP). Ruminally degradable protein is subject to degradation by the microbial population in the rumen and subsequent production of microbial cell protein (MCP). Ruminally undegradable protein can bypass the rumen and be used directly by the animal (Wickersham et al., 2003). Microbial cell protein assimilated in the rumen is a protein source for the animal, however it is usually of lower quality than dietary protein. Ruminal microbes can utilize non-protein nitrogen (NPN), such as urea, just as efficiently as a high quality dietary protein. Because NPN is cheaper than a true protein like casein, NPN is

often included in bovine rations. The advantage of feeding RUP is that a higher quality protein is made available for digestion in the small intestine with a greater benefit to the host. However, this feeding practice is only advantageous if the protein requirement of the animal is not being met. In contrast, a means where protein is protected from digestion in the small intestine could be applied in the horse. Instead of meeting the protein requirements of the horse, this product would be designed to supply N to the hindgut, which would be advantageous where if there are situations where a lack of N is limiting to the microflora. A protein protected from enzymatic digestion in the small intestine could ensure that the microbial population of the hindgut are provided with sufficient N.

Several researchers in the 1970's conducted studies to evaluate the response of protein and NPN supplementation in the horse. When urea was fed to cecally cannulated ponies, microbial protein synthesis and cellulose digestion in the hindgut was stimulated (Nelson and Tyznik, 1971). More recently, data from research done by Santos et al. (2012) indicated that the microbial population in the cecum can utilize casein and urea as nitrogen sources, and researchers saw greater microbial growth when supplementing casein compared to urea (Santos et al., 2012). However, feeding excessive urea is detrimental to ponies (125 to 136 kg), as those fed 450 g succumbed to ammonia toxicity (Hintz et al., 1970).

Hindgut Fermentation

The hindgut, comprised of the cecum and colon, is the major site of microbial fermentation in the equine. The vast microbial population that inhabits this area is key to proper digestion and absorption of nutrients in the horse (Kern et al., 1974; De Fombelle et al., 2003). Prior to reaching the hindgut, a large proportion of protein and carbohydrates is digested in the

small intestine, however structural polysaccharides and fructans reach the hindgut intact as they are resistant to endogenous enzymes of the small intestine.

Fermentation of Fiber in the Hindgut

Carbohydrates are traditionally classified based on location within the plant (Van Soest, 1994). Cellular contents, or non-structural carbohydrates (NSC), are comprised of hydrolyzable carbohydrates (CHO-H) and nonhydrolyzable, but rapidly fermentable carbohydrates (CHO-F_R). In the horse, much of the CHO-H is digested in the small intestine. But when the capacity of the small intestine is exceeded, these simple carbohydrates enter the hindgut and undergo rapid microbial fermentation (Hoffman et al., 2001). Although resistant to enzymatic fermentation, soluble fiber is rapidly fermented in the hindgut like that of starch. For this reason, Hoffman et al. (2001) proposed a new carbohydrate partitioning system specific to the horse, grouping carbohydrates into 3 main categories. These categories include carbohydrates that undergo hydrolysis in the small intestine (CHO-H), slowly fermentable carbohydrates such as NDF and ADF (CHO-F_S), and carbohydrates that escape the small intestine but are rapidly fermentable (CHO-F_R; Fig 1.1). When CHO-F_S and CHO-F_R reach the hindgut, they undergo microbial fermentation to produce VFA (Daly et al., 2012).

VFA are then absorbed and can provide as much as 70% of the horse's energy needs (Bergman, 1990). Similarly, in the rumen, fiber fermentation yields VFA and protein to the host in the form of microbial cell protein (Varga, 1997). Bacteria, fungi, and protozoa make up the group of microorganisms responsible for degradation of fiber in the rumen and hindgut. Although little is known about the microbial population of the hindgut, these organisms function similarly to those that inhabit the rumen (Costa and Weese, 2012).

Role of Cellulolytic Bacteria

Carbohydrate fractions that flow into the cecum are subject to digestion by the resident microbial population. Microbes adhere to the particles and secrete enzymes that begin the digestion progress. Although fungi and protozoa are involved in degradation of fiber in the hindgut, bacteria are largely responsible for microbial fermentation. Carbohydrate digesting bacteria can be classified as lactate-utilizing (LUB), cellulolytic, or amylolytic. Diet plays an integral role in types of microorganisms within bacterial populations. Dietary changes are generally associated with vast alterations in the microbiome (de Fombelle et al., 2003). Decreased fiber fermentation has been reported in horses with an increase in dietary starch (de Fombelle et al., 2003). The hindgut's microbial population in horses fed a diet high in concentrates favors the proliferation of lactic acid-producing (LAB) and amylolytic bacteria. As lactic acid is produced, pH declines, thus leading to decreased fibrolytic and LUB (Daly et al., 2002; de Fombelle et al., 2003, Julliand and Grimm, 2016).

In addition to diet, location within the hindgut has an effect on the makeup of the microbial population. While both the cecum and colon are primary sites of cellulolytic activity, greater concentrations of cellulolytic microorganisms are present in the cecum when compared to the colon (Kern et al., 1974; De Fombelle et al., 2003). de Fombelle et al. (2003) demonstrated a greater proportion of cellulolytic bacteria in the cecum compared to the colon. These authors concluded that the cecum is a more favorable site of cellulolytic activity. Regardless of site, cellulolytic bacteria only make up a small proportion of the total anaerobic bacterial population in the digestive tract (Julliand et al., 1999; Kern et al., 1974).

The majority of fibrolytic bacteria include *Fibrobacter succinogens*, *Ruminococcus flavefaciens*, and *Ruinococcus albus* (Davies, 1964). *Ruminococcus flavefaciencs* appears to be the most prolific species identified in the cecum (Julliand et al., 1999). These species are similar of those found in the rumen, although in different concentrations. Julliand et al. (1999) observed a 100-fold decrease in total and cellulolytic concentrations within the cecum compared to ruminal contents.

Fibrolytic species are generally considered be acid intolerant and their growth is suppressed in acidic conditions. At a low ruminal pH (< 6.2), fiber degradation in suppressed in cattle (Hoover, 1986). Similar findings were observed in the equine. Horses consuming a diet high in CHO-H or CHO-F_R experienced depressed growth of fibrolytic bacteria and subsequent fiber disappearance (de Fombelle et al., 2001; Daly et al., 2012).

Volatile Fatty Acids

Sugars produced from microbial degradation of fibrous components such as cellulose and hemicellulose can be hydrolyzed to form VFA. Volatile fatty acids are the major end products of microbial fermentation in the hindgut. Once polysaccharides are broken down into monosaccharides, glycolysis can occur via the Embden-Meyerhoff pathway and is responsible for catabolizing monosaccharides into 2 molecules of pyruvate. Microbes can then ferment pyruvate into several different products, including the VFAs acetate, propionate, and butyrate. Additionally, lactate, methane (CH_4), hydrogen (H_2) and carbon dioxide (CO_2) are produced (Hobson and Stewart, 1991; Shirazi-Beechey, 2007).

The cecal VFA profile is dependent upon the type of substrate consumed. Acetate is the most predominant VFA produced and usually accounts for 55 to 90% of the total VFA

concentration. Production of acetate is positively associated with fiber digestion as it is a major product of fiber-fermenting bacteria. Acetate production is also positively related to CH_4 production as H_2 and O_2 are produced.

Propionate increases in both ruminants and equines being fed a high grain diet as it is a product of LAB. Hydrolyzable carbohydrates present in grain are fermented by LAB, resulting in the production of propionate (De Fombelle et al., 2001; Allison, 1969). By contributing to glucose synthesis directly through the supply of carbon atoms, propionate is considered a major gluconeogenic substrate (den Besten et al., 2013).

The third most predominant VFA in both the rumen and cecum is butyrate. Butyrate is another product of lactate fermentation, but can also be converted from acetate. Production of butyrate is used as an energy substrate for epithelial cells, contributing to stronger epithelium. In ruminants, butyrate stimulates ruminal papillae growth leading to increased surface area for absorption of nutrients (Daniels and Yohe, 2014).

Unlike the small intestine, the surface of the large intestine has no villi. Instead it contains crypts that are lined with columnar epithelial cells. These cells contain colonocytes that are vital to absorption of VFA. The primary mechanism involved in VFA absorption is diffusion across the luminal and basolateral membrane after the molecule has become protonated and thus is lipid soluble. This is linked to the Na^+ H^+ exchange and is advantageous to the animal because it requires less energy input. The monocarboxylate transporter (MCT-1) is responsible for transporting 1 ionized VFA molecule out of the cell, across the basolateral membrane. This action is facilitated through the exchange of a bicarbonate (HCO_3-) molecule for 1 VFA molecule. The presence of VFA in the lumen of the large intestine also aid in gastrointestinal tract (GIT) regulation as they stimulate absorption of sodium (Na^+), chloride, (Cl^-), and water

(H_2O) and the secretion of bicarbonate (HCO^3-) using 2 carrier-mediated transporters: SMCT-1 and SLC5a8 (Stein et al., 2000; Daly et al., 2011).

Effect of Protein on Fiber Digestion

Slyter et al. (1979) determined that a concentration requirement of 2 to 5 mg NH_3-N per 100 ml of rumen fluid was sufficient for maximum growth of rumen microbes. The microbial requirement for ammonia is met from both exogenous sources such as feed and endogenous sources including salvia, blood, and sloughed ruminal epithelial cells. Nitrogen required for microbial growth comes from both peptides and ammonia. In fact, ammonia is the preferred substrate for most fiber fermenting bacteria (Allison, 1969). Fiber digesting bacteria use ammonia and CO_2 as growth factors which can stimulate fiber degradation.

By supplementing nitrogen in the form of protein or NPN, gut microbiota may increase their activity and/or populations. In turn, digestibility of lower quality forages can be improved, creating a positive associative effect. Because of the readily available native forage in the Midwest, particularly Kansas and Oklahoma, it is necessary to maximize forage utilization and animal performance, while keeping profitability in mind.

Voluntary DMI has been shown to decrease in cattle fed forages with < 7% CP (Moore and Kunkle, 1995). In several studies in cattle, researchers observed increases in DMI as well as DM and fiber digestibility of low quality hay (< 7% CP) due to protein supplementation (DelCurto et al., 1987; Hannah et al., 1990; Köster et al., 1995). Researchers saw increased ($P <$ 0.01) DM digestibility of dormant Bluestem grass supplemented with 3 increasing levels of CP (13%,17.5%, 26%) in ruminally cannulated steers. Additionally, authors saw increased fiber digestion in cattle who were supplemented with the medium and high treatments. In contrast,

fiber digestion was decreased in cattle supplemented with the lowest CP treatment compared to the control (Hannah et al., 1990). Hannah et al. (1990) concluded that supplementing a low-quality roughage with at least 20% CP (DM basis) can increase intake and substrate utilization. Similarly, sheep fed soybean stovers had increased ($P < 0.05$) DM and NDF digestibility when alfalfa was added to the diet compared to the control (Soofi et al., 1982).

Supplemental protein may not influence forage intake and digestion in cattle being fed forages containing > 7% CP (McCollum and Horn, 1990; Minson, 1990). It is likely that forages that are < 7% CP are unable to satisfy the nitrogen requirement of ruminal microbes. In cattle fed a greater CP containing forage, fiber fermenting microbes are provided with sufficient substrate for growth and function. Increased fiber digestion due to nitrogen supplementation is associated with an increase in rate of passage and subsequent increase in forage intake (Köster et al., 1996; McCollum and Galyean, 1985).

Little work has been done in the equine to evaluate the effect of protein supplementation on fiber utilization. In 1 study, small amounts of dietary urea reached the large intestine. Subsequently, cellulose degradation was increased (Nelson and Tyznik, 1967). The NRC (1989) reported that urea can comprise up to 4% of the total diet. Elevated urea in the blood results in excretion into the hindgut, thus having a potential benefit to microbial metabolism (Nelson and Tyznik, 1971; Prior et al., 1974).

In an unpublished *in vitro* study conducted at Kansas State University, researchers saw an increase ($P < 0.01$) in DM, NDF, and ADF disappearance when sodium caseinate was added to bottles containing native grass and cecal inoculant. In the same study, sodium caseinate had no effect on substrate disappearance in bottles containing alfalfa. Results from this study concur with previous studies that showed increases in low-quality forage digestion with protein

supplementation in cattle (McCollum and Horn, 1990; Köster et al., 1996). This may implicate the benefit to supplementing nitrogen to microbes of the cecum when poor quality forage is fed in order to increase efficiency of fermentation.

Sodium Caseinate

Sodium caseinate is a combination of casein and sodium hydroxide and accounts for a large majority of proteins found in milk. Casein is heat stable, highly soluble, and contains a large proportion of essential amino acids (EAA). Because of these properties, sodium caseinate can be used as a high-quality protein source in livestock diets.

Much work with sodium caseinate has been conducted in dairy cattle where feeding a ruminally-protected casein has been shown to improve milk production (Clark et al., 1977; Rooke et al., 1987). Post-ruminal infusions of sodium caseinate increased milk yield and milk protein in lactating Holsteins (Spires et al., 1975). As a high-quality protein, sodium caseinate can be broken down and eventually synthesized into glucose providing a substrate necessary for milk synthesis.

Casein contains highly soluble, intact AA, and increased microbial growth in both ruminal and cecal cultures (Santos et al, 2012; Maeng et al, 1976). When infused into the rumen, sodium caseinate elicited an increase in OM intake in cattle fed a low-quality (1.9% CP) forage. Additionally, ruminal pH decreased with increasing levels of sodium caseinate (Köster et al., 1996).

It can be theorized that if the equine hindgut is in a low nitrogen environment, supplementation with sodium caseinate could enhance fiber digestion through increased microbial activity and/or fiber fermenting bacterial populations. However, because protein is

primarily digested in the small intestine of the horse, this hypothesis has not yet been evaluated. Therefore, a mode whereby protein reaches the hindgut intact is essential to ensure supplementation. This may be accomplished through encapsulation utilizing fat as a hydrogenated vegetable oil has been used to protect sodium bicarbonate from the small intestine in transit to the cecum (Pagan et al., 2007). However, this product should be studied further to confirm efficacy as means to protect products from small intestine absorption.

Digestibility Markers

The most accurate measurement of total tract digestibility is made through total fecal and urine collection (Miraglia et al., 1999). However, this method can be potentially time consuming and price prohibitive. Harnesses that collect urine and feces are expensive to purchase and require additional time to train the horse. Without these harnesses, it is difficult to collect total output of the horse without contamination of urine and feces. Moreover, unless monitored around the clock, feces have the potential to be stepped on and further contaminated by water or feedstuffs (Sales, 2012). With these difficulties in mind, several internal markers have been studied as effective means of predicting apparent digestibility in the horse.

Naturally occurring indigestible markers such as acid insoluble ash (AIA) and acid detergent lignin (ADL) are relatively easy use as they require no additions to the diet. When comparing AIA and ADL digestibility values to those generated with total fecal collection, AIA values are more similar while ADL values are consistently lower than total collection in the equine (Miraglia et al., 1999). Acid insoluble ash was first used to determine digestibility of diets fed to sheep (Van Keulen and Young, 1977). In a study performed to determine digestibility of cattle rations, AIA had a greater fecal recovery than ADL (Thonney et al., 1985). Because of

inconsistent recovery and low digestibility values, ADL has been classified as an unsuitable marker by many (Titgemeyer, 1997). Problems with using AIA also have been reported as grains contain insufficient concentrations to be measured. However, in high forage diets, nearly complete recovery has been shown (Van Keulen and Young, 1977; Sunvold and Cochran, 1991).

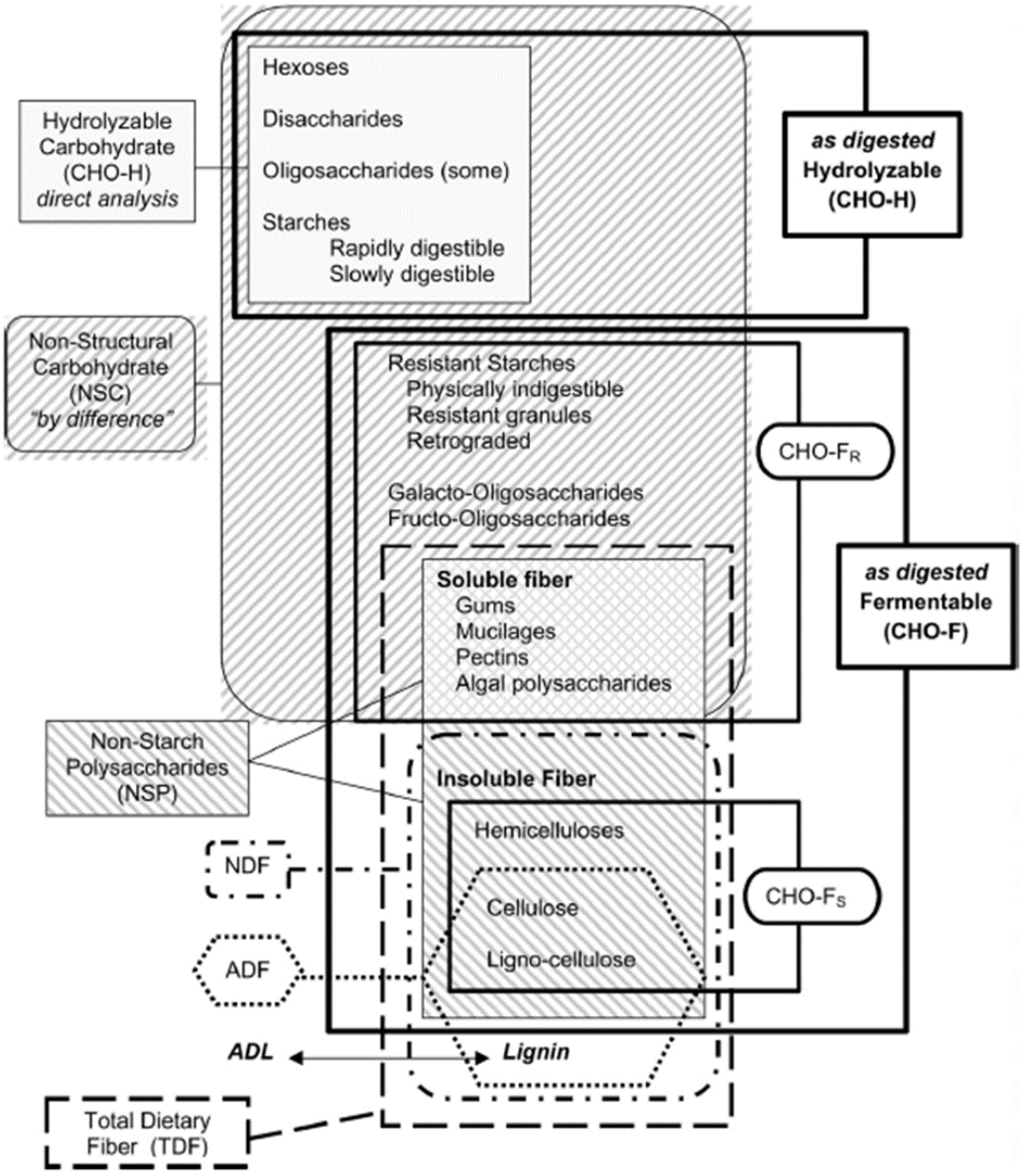
Acid detergent insoluble ash (ADIA) has also been used a natural, internal marker. Unfortunately, there is little published literature comparing this method to others. Bodine et al., 2002 concluded that ADIA content could potentially by increased by sand and soil ingestion in cattle, but the marker had recovery rates of 99.3% and 97.5% in 2 trials.

Regardless of the choice of marker, several problems can be encountered including incomplete recovery and collection of unrepresentative samples. Careful consideration of diet and animal should be accounted for before selecting an internal marker.

Summary

A nitogen-limiting environment can lead to decreased fermentative activity and reduced microbial growth (Hoover, 1986). Through protein supplementation, fiber digestion and DMI can be increased in cattle consuming low quality forages (< 7% CP; McCollum and Horn, 1990). The same nitogen-limiting situation could occur in the cecum of the horse as dietary protein is absorbed primarily in the small intestine. Therefore, this study was conducted to evaluate the effects of cecally administered sodium caseinate on equine hindgut fermentation and fiber digestion.

Figure 1.1 A carbohydrate partitioning system specific to the equine (Hoffman et al., 2011)



Chapter 2 - Effect of sodium caseinate of hindgut fermentation and fiber digestion in the equine

K.V. Jordan, J.S. Drouillard, T.L. Douthit, J.M. Lattimer

Kansas State University

Manhattan, KS 66506-1660

Abstract

Eight cecally cannulated Quarter Horses were used in replicated 4 x 4 Latin square design conducted in 4, 14-d periods to determine effects of sodium caseinate on equine hindgut fermentation and fiber digestion. Horses were assigned to 1 of 4 treatments during each period, consisting of control (water; CON), 0.125 g casein/kg BW (LOW), 0.25 g casein/kg BW (MED), or 0.5 g casein/kg BW (HI). Casein was solubilized in 800 mL of water and dosed directly into the cecum at 0700 and 1900 using a metal dosing syringe. Smooth Bromegrass hay (CP 8.50%) and water were available *ad libitum.* New hay was fed at 0700 and 1900 and orts were recorded at 1900 each day. During the final 3 d of each period, cecal digesta were collected every 6 h. Digesta pH was measured immediately after sampling, and samples were then frozen for subsequent analyses of VFA and NH_3 concentrations. Feed intake during the final 4-d of each period was recorded, and feces were collected over the 3-d sampling period, pooled, subsampled, and analyzed to determine pH, and digestibilities of DM, OM, NDF, and ADF. Digestibilities of DM, OM, NDF, and ADF were unaffected by treatment ($P > 0.40$). Cecal digesta pH was greater for horses dosed with CON and MED compared to horses receiving the LOW and HI treatments ($P < 0.01$). Cecal NH_3 concentrations increased linearly in response to the amount of casein administered ($P < 0.01$), and decreased 6 h after dosing and addition of new hay regardless of treatment ($P < 0.01$). Concentrations of VFA were unaffected by treatment, but VFA concentrations did change over time with the greatest concentrations observed 6 h after treatment and introduction of new hay ($P < 0.01$). Treatment did not affect DMI ($P \geq 0.17$). In this experiment, cecal infusions of sodium caseinate had no effect on fermentation parameters or fiber degradation. While a type II error may have occurred due to the small population size, it is more likely that the medium quality hay fed to these horses provided sufficient protein for microbial fermentation.

Introduction

In ruminants, supplemental dietary intake protein (DIP) optimizes the utilization of low quality forages (Köster et al., 1996). Both protein and NPN have improved fiber digestion and DM intake in cattle consuming forages that contain less than 7% CP (Moore and Kunkle, 1995; McCullum and Horne, 1990; Koster et al., 1995). By supplementing nitrogen to the microbial population of the rumen, fibrolytic bacteria can utilize peptides, amino acids, and ammonia for growth and ultimately to improve fermentation of feedstuffs and animal performance. Both the rumen and hindgut of the equine house vast microbial populations essential to the proper digestion and fermentation of feedstuffs. Although growth performance is not of great concern in the horse industry, proper digestion of fiber is critical to gut health and overall well-being. Consumption of low quality forages is generally associated with poor degradation, which may lead to impaction colic and inadequate nutrient availability. Because the small intestine in located before the primary site of fermentation in the horse, much of the dietary protein is absorbed before reaching the microbial population of the hindgut.

As in the ruminant, a protein-limiting rumen can result in decreased fiber digestion through diminished fermentative activity and/or reduced microbial growth. In theory, the same could be true in the cecum of the horse. However, this hypothesis is relatively unexplored. Thus, this study was designed to evaluate the effects of cecally infused protein, in the form of sodium caseinate, on fiber digestibility and cecal fermentation.

Materials and Methods

Animals, Facilities, and Diets

All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee. Eight mature Quarter Horses (4 geldings, 4 mares) with an initial mean BW of 515 ± 15.4 kg and previously fitted with cecal cannulae (Beard et al., 2011) were used in a replicated 4 x 4 (treatment x horse) Latin square design (Fig 2.1).

Horses were individually housed in 3.05 m x 3.66 m stalls bedded with pine shavings and turned out for 15 to 30 min per d onto a dry lot during non-collection days. Water and Smooth Bromegrass hay (Table 2.1) were provided *ad libitum*. Daily refusals of hay were recorded in the evening (1900 h).

Treatments

Treatments consisted of a control (CON; water only), 0.125 g sodium caseinate/kg BW (Low; Erie Foods International, Inc., Rochelle, IL), 0.25 g sodium caseinate/kg BW (MED), and 0.50 g sodium caseinate/kg BW (HI). Sodium caseinate was solubilized in 800 mL of distilled water using a 1-gallon heavy duty blender (Waring Commercial, Torrington, CT). Solutions were prepared daily and kept chilled (2^{o} C) until time of application when they were brought to room temperature and administered via the cecal cannulae using a 500-mL dosing syringe. Horses were dosed with the designated treatments twice daily (0700 and 1900 h) for 14 d. At the conclusion of each period, horses were switched to their next respective treatment (Table 2.2).

Sample Collection

Starting on d 12 of each period, cecal digesta was collected via gravity flow every 6 h (0100, 0700, 1300, 1900) for 3 d (d 12, 13, and 14). Samples were immediately strained through

4 layers of cheesecloth, placed into 500 mL containers (Specimen Storage Containers, #14955117A, Fisher Scientific, Pittsburg, PA), and frozen for later analyses. When collections overlapped with dosing, cecal digesta was collected before treatments were administered. Total fecal output was collected from the stall floor following defecation for 72 h starting on d 12. Prior to fecal collections, stalls were stripped of pine shavings and cleaned to avoid contamination of fecal contents. At the conclusion of the 72-h sampling period, feces were mixed by hand and a subsample was obtained. Fecal samples were frozen (-18° C) for later analyses

Sample Analyses

Strained cecal fluid was immediately analyzed for pH following collections using a portable pH meter (Thermo Scientific Orion 3 Star Portable pH Meter, Waltham, MA). Four, 1 mL aliquots of cecal fluid were pipetted into microcentrifuge tubes and deproteinated with 25% (wt/vol) meta-phosphoric acid. Samples were then frozen (-18° C) and saved for later analysis of VFA and ammonia.

Deproteinated cecal samples were thawed and centrifuged at 17,000 x *g* for 15 min. The aqueous supernatant was transferred to gas chromatography vials in duplicate and analyzed for VFA concentrations using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a DB-WAX capillary column (30 m x 0.53 mm x 0.5 mm film thickness; Sigma Aldrich, St. Louis, MO) and flame ionization detector. Hydrogen was used as a carrier gas at a flow rate of 22 cm/s, with a 1-µL split injection and a split flow of 50:1. The initial oven temperature of 80° C was increased by 10° C/min to 220° C. Inlet and detector temperatures were 250° C. Volatile fatty acids were quantified by comparison to known standards (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO) containing

acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate, caproate and heptanoate.

Deproteinized cecal samples were also analyzed, in duplicate, for NH_3 concentrations using a Technicon AutoAnalyzer3 (Technicon Instruments Corporation, Tarrytown, NY). Techniques used were in accordance to Technicon Industrial Method #512-77T and Broderick and Kang (1980).

Thawed fecal samples were dried at 55° C using a forced air oven for 24 h. Samples were air-equilibrated and then weighed. Partial DM was calculated as follows:

$$\frac{(Dry\ sample\ and\ pan\ weight) - Pan\ weight}{Wet\ sample\ weight} \times 100$$

Hay and fecal samples were then ground using a Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) until they passed through a 1-mm screen. A 1-g aliquot of each ground sample was used to determine DM, ash, and OM according to the protocols of the National Forage Testing Association (Undersander et al., 1993).

Neutral detergent fiber and non-sequential ADF concentrations in hay and fecal matter were determined using an ANKOM$^{200/220}$ Fiber Analyzer (ANKOM Technology, Macedon, NY) using the protocols established by Van Soest et al. (1991). Approximately 0.5 g (±0.05g) of ground hay and feces were weighed, in duplicate, into F57 filter bags (ANKOM Technology, Macedon, NY). Bags were sealed and samples were spread uniformly inside filter bags. Bags were suspended in the digestion apparatus containing 1,200 mL neutral detergent solution, 20 g sodium sulfite, and 4.0 mL of heat stable alpha amylase. Bags were agitated and heated for 75 m. The device was turned off and solution exhausted from the apparatus. Bags were rinsed 3 times,

using 2000 mL hot, deionized water for 5 min. During the first and second rinses, 4.0 mL of alpha amylase was also added. After rinsing, bags were removed of excess water and soaked in acetone for 3 min. Once the acetone had evaporated, bags were dried in an oven (105° C) for 12 h. Bags were then removed, placed into desiccators, cooled to room temperature, and weighed. Neutral detergent DM percentage was determined using the following equation:

$$\frac{\left(Weight\ of\ bag\ after\ digestion\ -\left(Bag\ weight\ \times\ Blank\ bag\ correction\right)\right)\ \times 100}{Sample\ weight\ \times DM}$$

To determine ADF concentration, bags were suspended in the digestion apparatus containing 2,000 mL acid detergent solution and agitated and heated for 60 min. Bags were rinsed 3 times, using 2,000 mL hot, deionized water for 5 min. After rinsing, bags were soaked in acetone for 3 minutes and dried (105° C for 12 h). Bags were then removed from the oven, placed into desiccators, cooled to room temperature, and weighed. Acid detergent fiber DM percentage was determined using the following equation:

$$\frac{\left(Weight\ of\ bag\ after\ digestion\ -\left(Bag\ weight\ \times\ Blank\ bag\ correction\right)\right)\ \times 100}{Sample\ weight\ \times DM}$$

Acid insoluble ash concentrations were determined according to the protocol established by Van Keulen and Young (1977). Acid insoluble ash was used as an indigestible marker to determine nutrient digestibility using the following equation:

$$100 - (100 \times \left(\frac{Nutrient\ in\ feed}{Nutrient\ in\ feces}\right) \times \left(\frac{Nutrient\ in\ feces}{Nutrient\ in\ feed}\right)$$

Statistical Analyses

All statistical analyses were performed using the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Digestibility and fecal pH were analyzed using fixed effects of treatment and period, with a random effect of horse. Cecal VFA, NH_3, and pH were analyzed using a random effect of horse and fixed effects of treatment, time, and treatment by time interaction. Dry matter intake was analyzed using the fixed effect of treatment, period, and treatment by period, with the random effect of horse. Degrees of freedom were determined using the Kenward-Rogers approximation. Significance was declared at $P \leq 0.05$ and a tendency considered at $0.05 < P \leq 0.10$. Differences among least-squares means were determined using the PDiff option of SAS.

Results

Cecal and Fecal pH

A treatment effect was detected on cecal pH (Fig 2.2). Cecal pH in horses dosed with the LOW and HI treatments, was less than the pH of control horses ($P < 0.01$). Mean cecal pH for the CON, LOW, MED, and HI treatment groups were 7.11, 7.04, 7.11, and 7.01 (SEM 0.05) respectively. Additionally, there was an effect of time on cecal pH (Fig 2.3). The lowest pH readings of the day were recorded at 0100 and 1300 compared to 0700 and 2100 ($P < 0.01$). Mean cecal pH for 0700, 1300, 1900, and 0100 were 7.22, 6.95, 7.16, and 6.93 (SEM 0.05), respectively. There was no treatment by time interaction detected. In the first treatment period, samples from the final collection time were not obtained and therefore, cecal pH data from those

8 samples were not included in the analysis. There were no treatment differences in fecal pH ($P > 0.10$; Fig 2.4).

Cecal VFA

Infusing casein had no effect on cecal concentrations of acetate, propionate, butyrate or A:P ($P > 0.10$; Fig 2.5, 2.6). Similarly, total VFA concentrations was unaffected ($P > 0.10$) by treatment (Fig 2.7). Valerate was only detected in 1 horse so a graph was excluded. Isovalerate was elevated ($P < 0.05$; Fig 2.8) in horses dosed with the HI treatment compared to horses receiving the lower dosages. Isobutyrate concentration was less in horses in the LOW treatment group, but concentration then increased linearly as casein level increased ($P < 0.05$; Fig 2.9). Caproate and heptanoate concentrations were detected in trace amounts and therefore were not included in the tables and figures.

Acetate, propionate, and butyrate concentrations increased at 1200 and 0100 when compared to 0700 and ($P < 0.05$; Fig 2.10). Additionally, there was an effect of time on cecal A:P ratio and total VFA concentration ($P < 0.01$) In the first treatment period, samples from the final day at 0100 were not obtained and therefore, VFA data from those 8 samples were not included in the analysis.

Digestibility

Treatment had no effect on digestibility of DM, NDF, ADF, and OM ($P > 0.10$; Table 2.3). When compared to the control, DM digestibility numerically decreased from 46.30% to 43.85% and 45.51% in horses on the LOW and HI treatments, respectively ($P > 0.10$). However, DM digestibility of horses in the MED treatment group numerically increased from the CON to

47.76%. Organic matter, NDF, and ADF digestibility followed the same trend as DM digestibility with digestibility numerically decreasing in the LOW and HI treatment groups when compared to the CON. Horses in the MED group had slight increases in NDF and ADF digestibility compared to the CON.

Cecal Ammonia Concentration

Cecal NH_3 concentration increased linearly as level of casein dosed was increased ($P < 0.01$; Fig 2.11). Means were 0.56, 0.71, 0.99, and 1.48 mM for the CON, LOW, MED, and HI groups, respectively. Additionally, there was an effect of time on NH_3 concentrations ($P < 0.01$; Fig 2.12), with concentrations being lowest ($P < 0.01$) 6 h after dosing.

Dry Matter Intake

Dry matter intake was unaffected regardless of treatment ($P \geq 0.17$; Fig 2.13).

Discussion

Although time of intake was not recorded, 0700 and 1900 were generally associated with the greatest dietary intake as horses were presented with new hay. Within 6 h of consumption, contents can reach the cecum (De Fombelle., 2004). Contents undergo microbial fermentation, which results in the production of VFA and lactate, thereby decreasing cecal pH. This was observed in the current study as there was an effect of time on acetate, propionate, and butyrate as summarized in Fig 2.5. For all 3 VFA, there was a significant increase in concentration at 1200 and 0100 compared to 0700 and 1900 ($P < 0.01$). Volatile fatty acid concentration was inversely related to pH, which is expected as the production of acid decreases pH.

While horses on the CON and MED treatment had greater mean cecal pH than those dosed with the LOW and HI treatments, it is important to note that, mean pH values were similar ranging from 7.01 to 7.11. This might be explained by inter-horse variability, so one must be careful in drawing conclusions regarding cecal pH based on treatment. Means were well within normal ranges usually observed in the horse (Geor et al., 2013).

When comparing mean fecal and cecal pH values, fecal values tended to remain lesser. Since greater concentrations of lactate-producing bacteria are found in feces, this finding was not unexpected. If fact, others have also reported decreased pH in feces compared to cecal material (Douthit et al., 2014). These findings also provide further evidence that fecal pH is not reflective of cecal parameters (Drougol et al., 2012).

Increased fiber degradation is usually associated the production of acetate, however because there were no differences in fiber digestibility, no differences in cecal acetate concentrations were expected, nor observed. Although cecal pH was greater in horses receiving the CON and MED treatment compared to horses receiving the LOW and HI treatments, these differences were not reflected in cecal VFA concentrations. While numerically VFA concentrations were increased in horses treated with the LOW and HI treatments, these were not statistically significant. Both acetate and propionate are major end products of fiber degradation, but as there were no differences in digestibility, no changes in VFA concentrations were expected or observed. Again, there was high inter-horse variability that may have masked treatment differences. Additionally, a smaller sample size contributed to higher standard errors observed for these parameters.

Cecal NH_3 concentration increased linearly with level of casein dosed was increased. ($P < 0.01$; Fig 2.7). Indeed, this is a result of fermentation as casein is a readily soluble protein. It

would be logical to assume that as more casein was added, more ammonia would be produced from the fermentation process and our results support this hypothesis. There is little information about ammonia concentration in the cecum of horses. In horses fed dietary casein, cecal NH_3 concentrations were greatest 1 h after feeding (10.8 mg/100 ml), but declined 6 h after feeding (5.0 mg/100 ml; Nelson and Tyznik, 1971). This aligns with our results in that concentrations were lowest 6 h after feeding and dosing. Intraruminal infusions of casein have resulted in similar increases in ruminal NH_3-N concentrations (Slyter et al., 1979; Köster et al., 1996).

There were no differences in digestibility of DM, NDF, ADF, and OM in this study. This contradicts previous in vitro data from our lab that showed an increase ($P < 0.05$) in DM, NDF, and ADF disappearance in native prairie hay supplemented with sodium caseinate. Reitnour and Salsbury (1972) findings support the current study when horses that were cecally infused with varying proteins (fishmeal, soybean meal, and linseed meal) had similar DM digestibilities among treatments when fed a basal diet containing 6.1% CP. Authors hypothesize that this was due to increased passage rate caused by distension, as an additional 600 mL of water required to administer the slurry (Reitnour and Salsbury, 1972). In the present study, 800 mL of water were used to administer treatments and in turn may have had an effect of passage rate. However, as demonstrated in cattle, supplemental protein can increase digestibility and DMI of low-quality feedstuffs, but will have no effect when the CP content of the hay fed is over 7% (McCollum and Horn, 1990; Minson 1990). Because the hay fed in the current study was of medium-quality and had a CP content of 8.50%, it is likely that the diet satisfied the fibrolytic microbes' needs for ammonia and truly have no effect on fiber digestion nor DMI.

Although digestibilities were not statistically different between treatments, numerical patterns did emerge. Horses in the LOW and HI treatment groups had lesser DM, NDF, and ADF

digestibility than horses in the CON and MED group. Alterations in cecal pH may explain why the LOW and MED treatments had numerically lower digestibility values as there may have been slight shifts in microbial populations. Previous studies in the horse resulted in similar NDF, ADF, and OM digestibilities, using AIA as an internal marker (Bergero et al., 2005). However, DM digestibility values for this study were slightly lower than observed in other experiments. Authors from one study reported ADF and NDF digestibility of a similar quality hay to be in the upper 20 percent which is much less than the values reported herein (Varloud et al., 2001). OM digestibility in the same study was 48 percent. Köster et al. (1996) utilized AIA as internal marker in cattle and observed NDF digestibilities around 55% in cattle intraruminally infused with casein. The hay fed in this study had a CP content of 8.5% (DM basis) which may have been sufficient alone for optimal fibrolytic performance in the cecum.

Conclusion

In summary, varying levels of sodium caseinate administered intracecally to horses consuming a medium quality bromegrass, had no effect on fermentation parameters or fiber degradation. Although numerical patterns were observed, more work needs to be conducted to establish if a type II error occurred due to a small population size or if an average quality grass hay supplies sufficient nitrogen to the microflora of the hindgut.

Limitations

Lack of treatment differences may have been attributed to the small sample size and variability in animals used as shown in the high standard error rates detected. By increasing sample size for future experiments, statistical power could be improved upon.

Figure 2.1 Experimental design

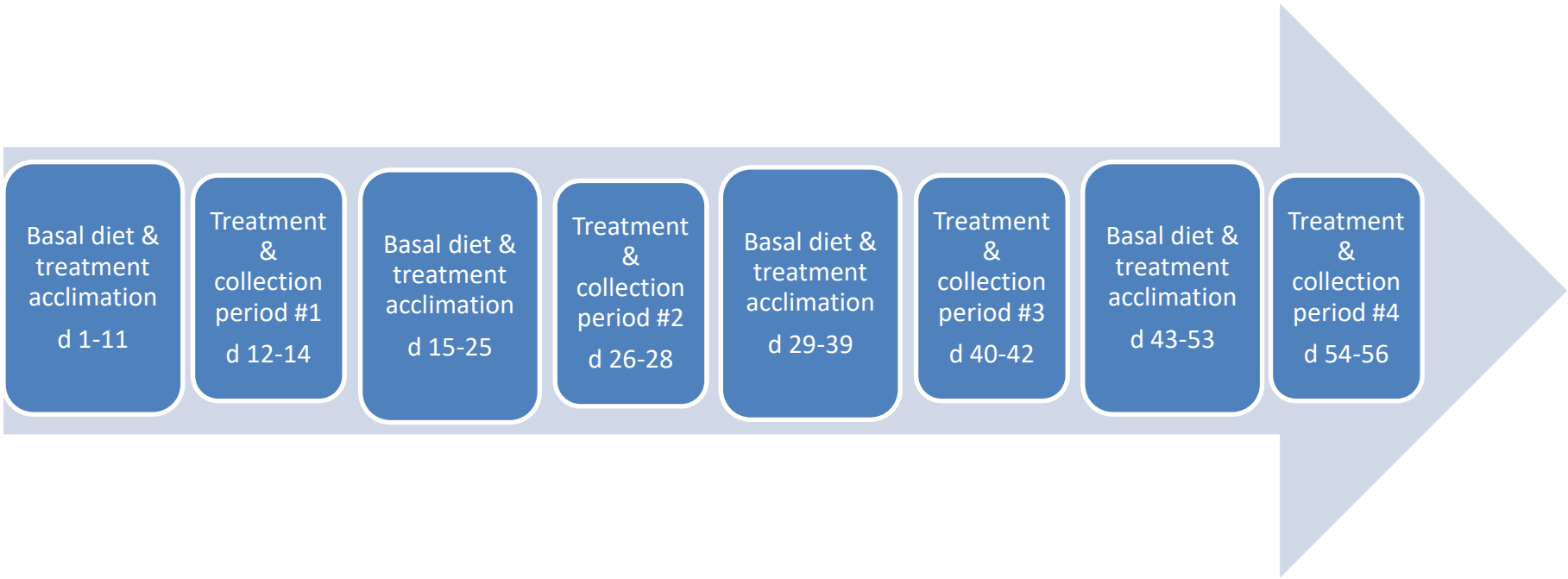


Table 2.1 Level of sodium caseinate administered by period[†]

Horse	Period 1	Period 2	Period 3	Period 4
1	0	0.125	0.25	0.50
2	0	0.125	0.25	0.50
3	0.125	0.25	0.50	0
4	0.125	0.25	0.50	0
5	0.25	0.50	0	0.125
6	0.25	0.50	0	0.125
7	0.50	0	0.125	0.25
8	0.50	0	0.125	0.25

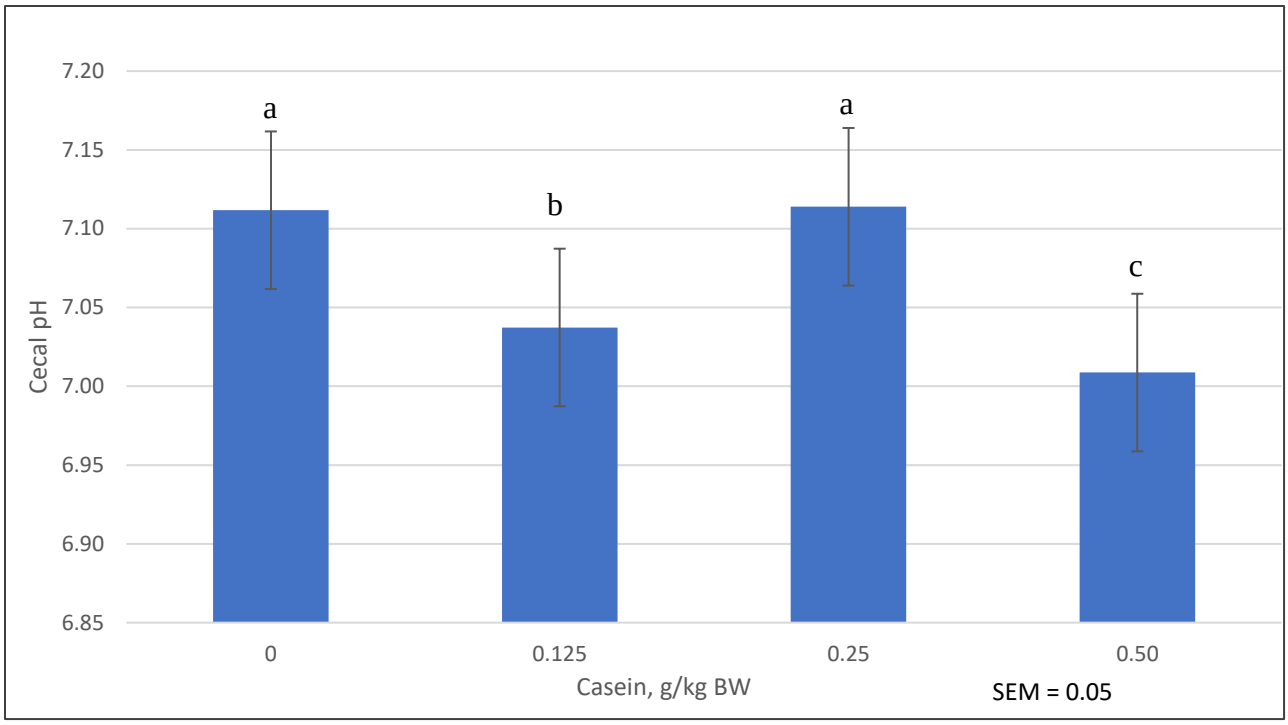
[†]Horses in the control group were administered 800 mL of distilled water via cecal cannulae. Horses in the low, medium, and high groups were administered 0.125, 0.25, and 0.50 g sodium caseinate/kg BW respectively, solubilized in 800 mL of distilled water via cecal cannulae. Treatments were administered 2x daily.

Table 2.2 Analysis of Smooth Bromegrass hay[†,‡]

Item, %	
DM	90.40
OM	91.38
CP	8.50
NDF	58.60
ADF	34.90
Non-fiber carbohydrates	20.30
Lignin	4.60
Crude fat	3.20
DE, Mcal/kg	2.59
Calcium	0.50
Phosphorus	0.15
Magnesium	0.11
Potassium	2.29
AIA	2.62

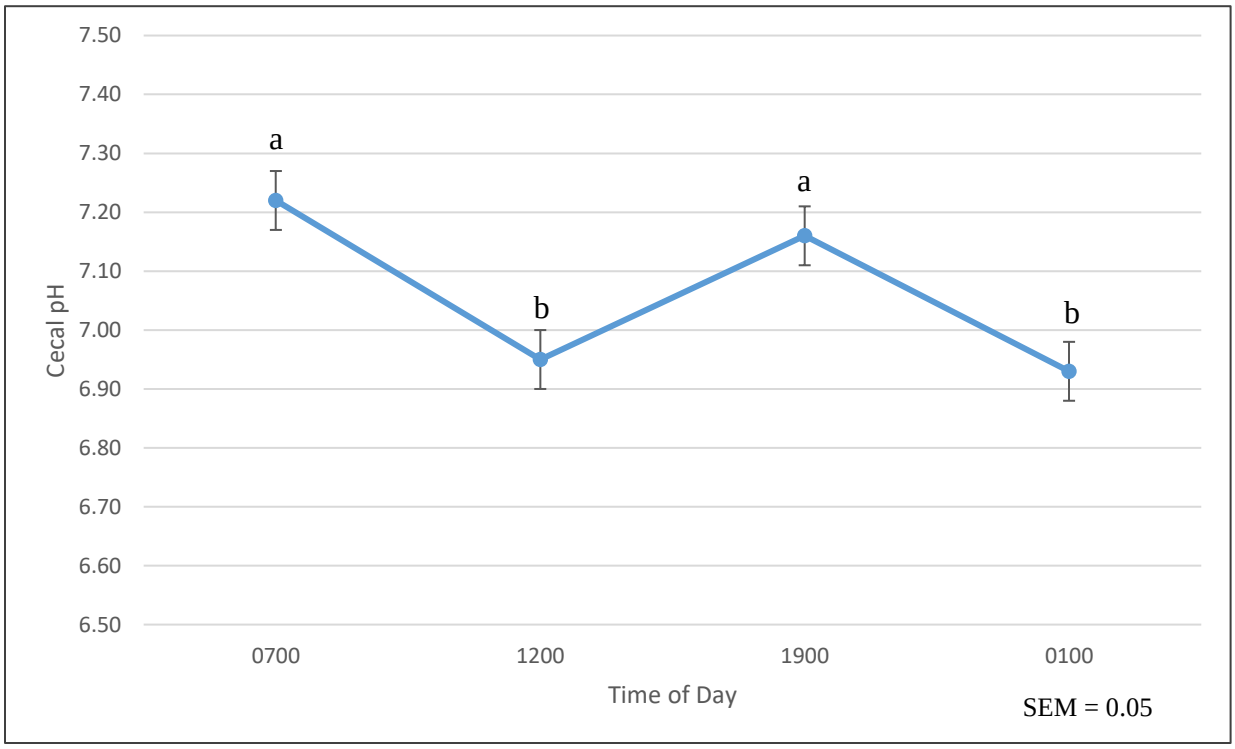
[†]Proximate analysis was determined using wet chemistry (Dairy One Forage Lab, Ithaca, NY)
‡Values on a DM basis

Figure 2.2 Effect of cecally infused sodium caseinate on cecal pH †, ‡



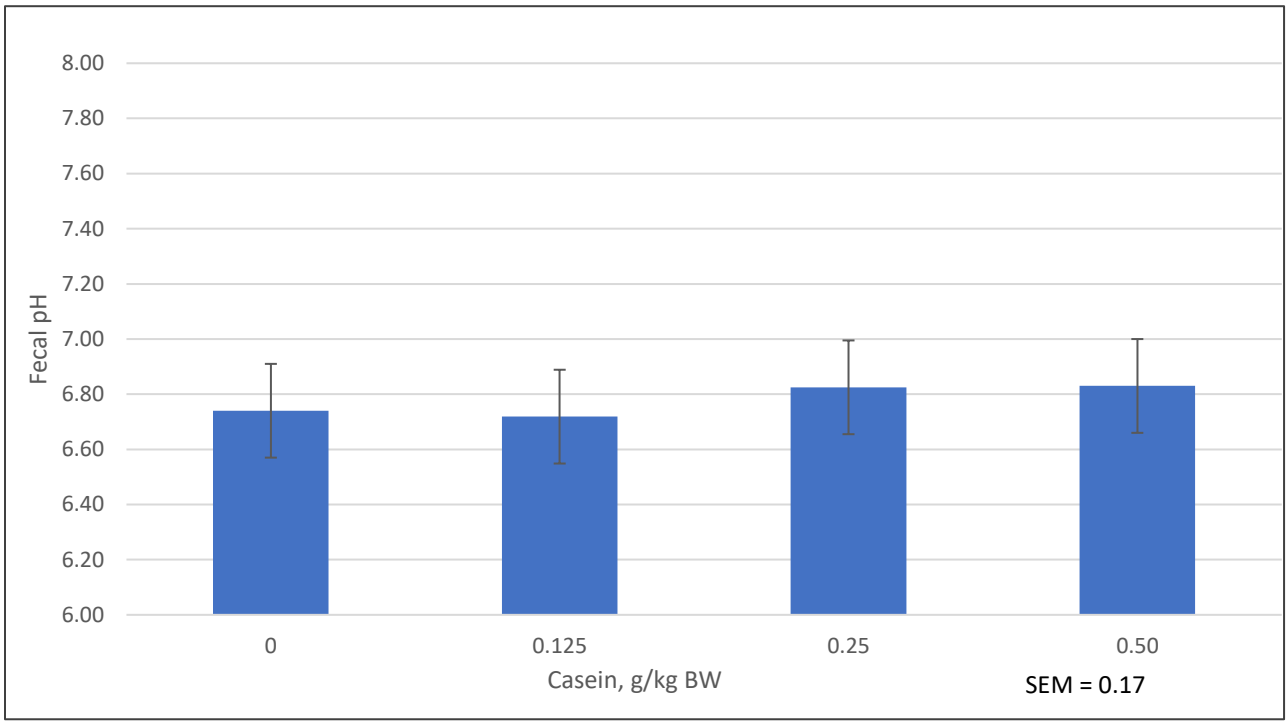
†Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g casein/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
‡ Values with different superscripts differ ($P < 0.01$)

Figure 2.3 Effect of time on cecal pH[†,‡]



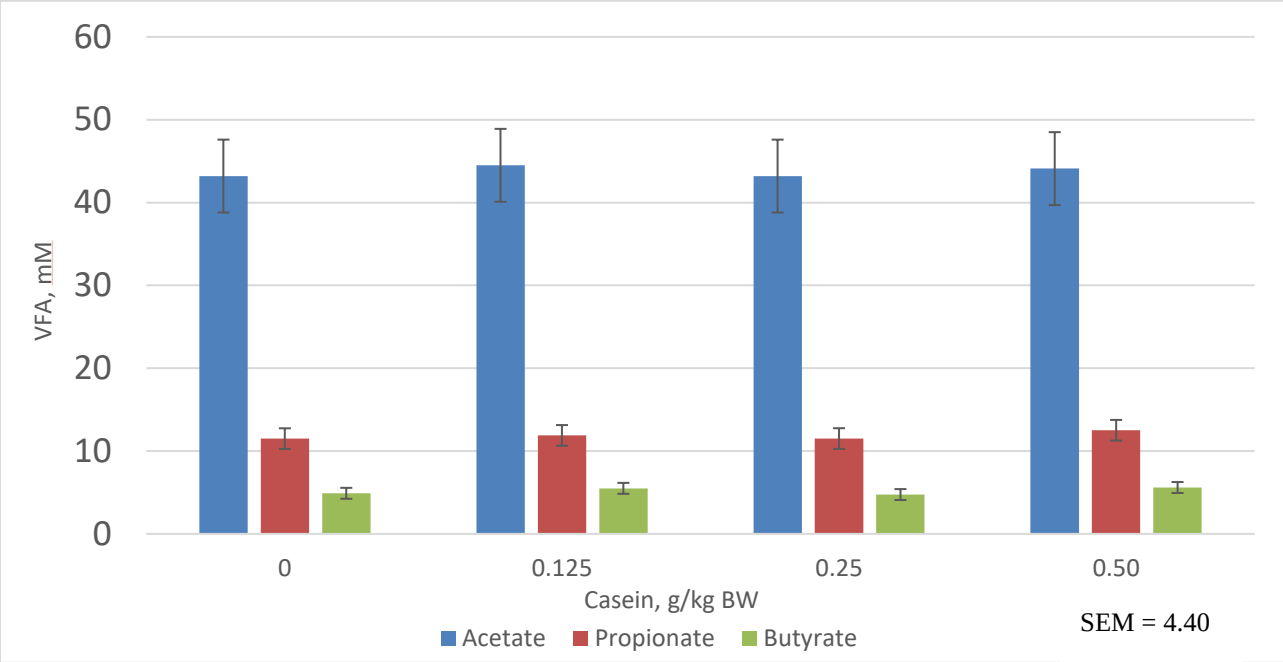
[†]Horses were provided *ad libitum* Smooth Bromegrass hay and water. New hay was offered at 0700 and 1900.
[‡]Means across all treatment groups
[a,b]Values with different superscripts differ ($P < 0.01$)

Figure 2.4 Effect of cecally infused casein on fecal pH[†, *, ‡]



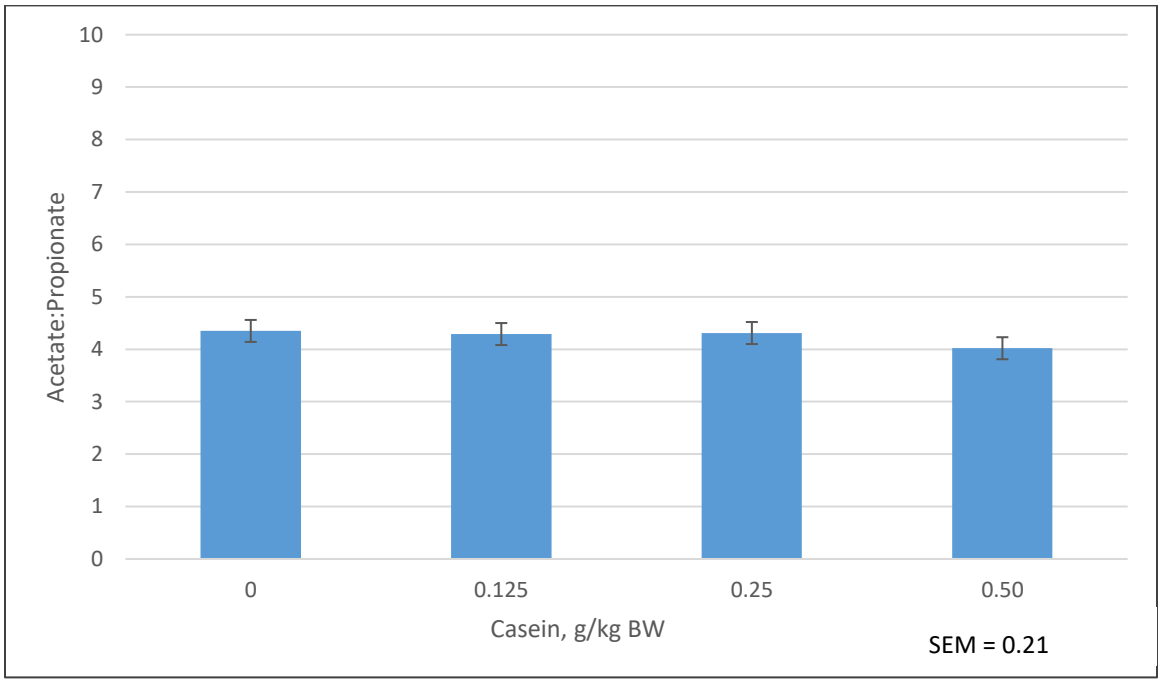
[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g/kg BW casein respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
*pH of pooled fecal samples representing d 12, 13, and 14 of each period.
[‡] No effect of treatment ($P > 0.10$)

Figure 2.5 Effect of cecally infused sodium caseinate on cecal VFA concentrations[†, ‡, ††]



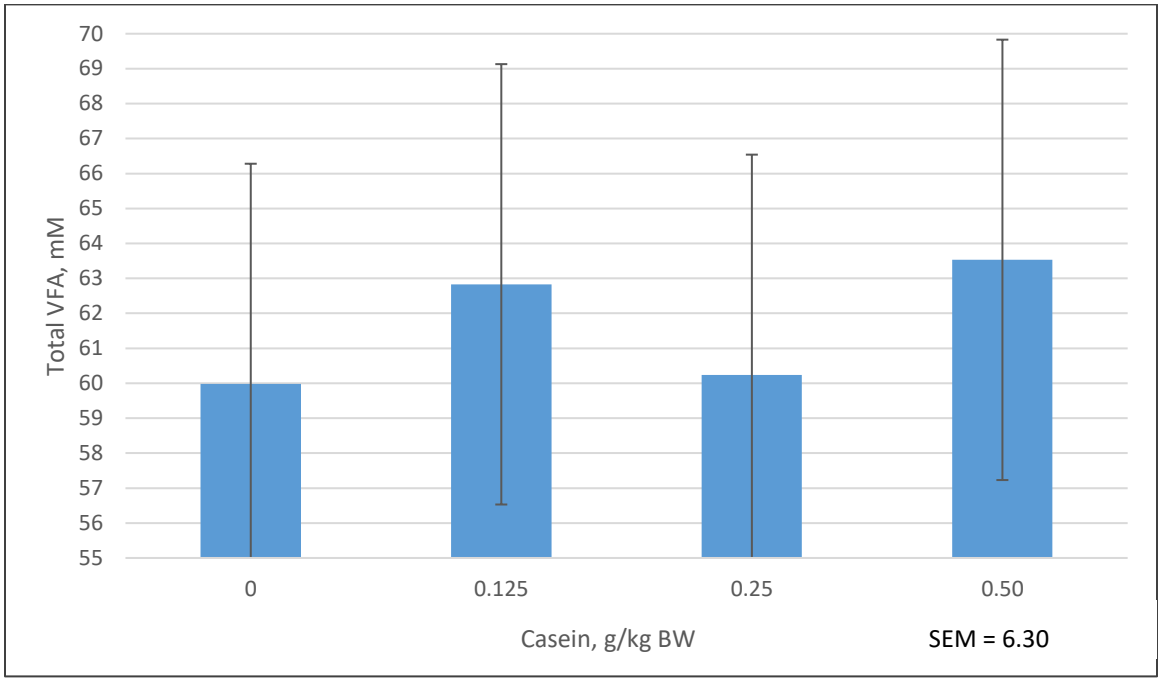
[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.50 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
[‡] No effect of treatment ($P > 0.10$)
[††]No treatment by time interaction ($P > 0.10$)

Figure 2.6 Effect of cecally infused sodium caseinate on cecal acetate:propionate ratio[†, ‡, ‡‡]



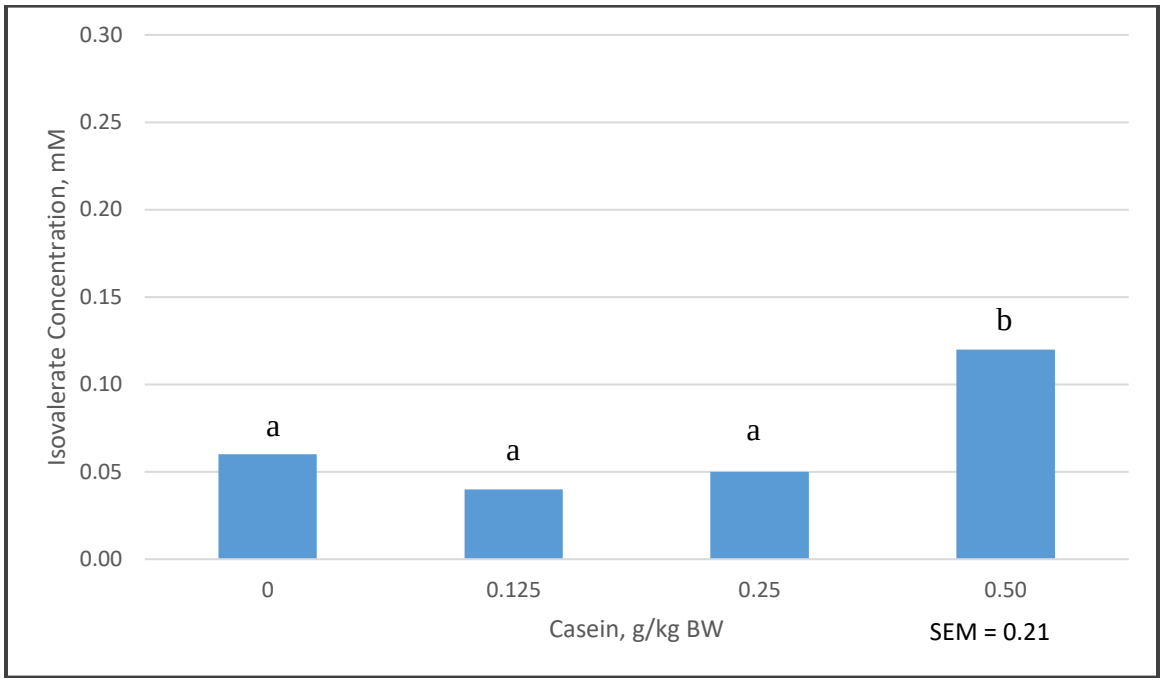
[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
[‡] No effect of treatment ($P > 0.10$)
[‡‡]No treatment by time interaction ($P > 0.10$)

Figure 2.7 Effect of cecally infused sodium caseinate on total cecal VFA concentrations[†,‡,‡‡]



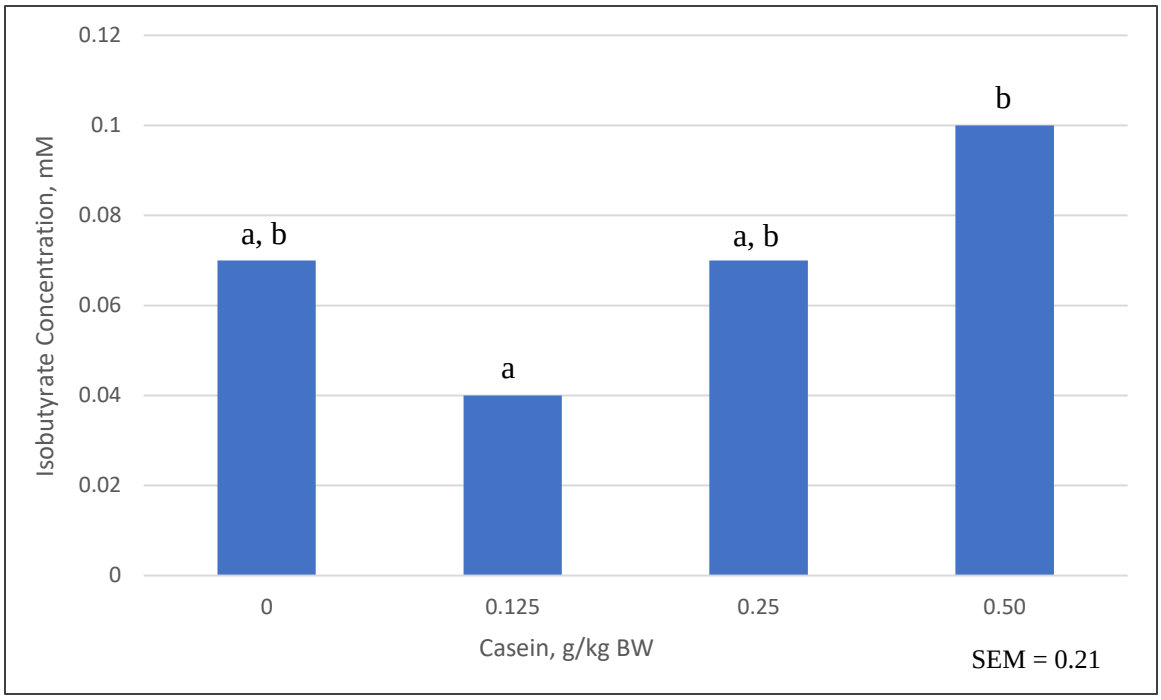
[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannula. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannula.
[‡] No effect of treatment ($P > 0.10$)
[‡‡] No treatment by time interaction ($P > 0.10$)

Figure 2.8 Effect of cecally infused sodium caseinate on cecal isovalerate concentrations[†,‡,‡‡]



[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
[a,b]Bars with different superscripts differ ($P < 0.05$)
[‡‡]No treatment by time interaction ($P > 0.10$)

Figure 2.9 Effect of cecally infused sodium caseinate on isobutyrate concentrations[†,‡]

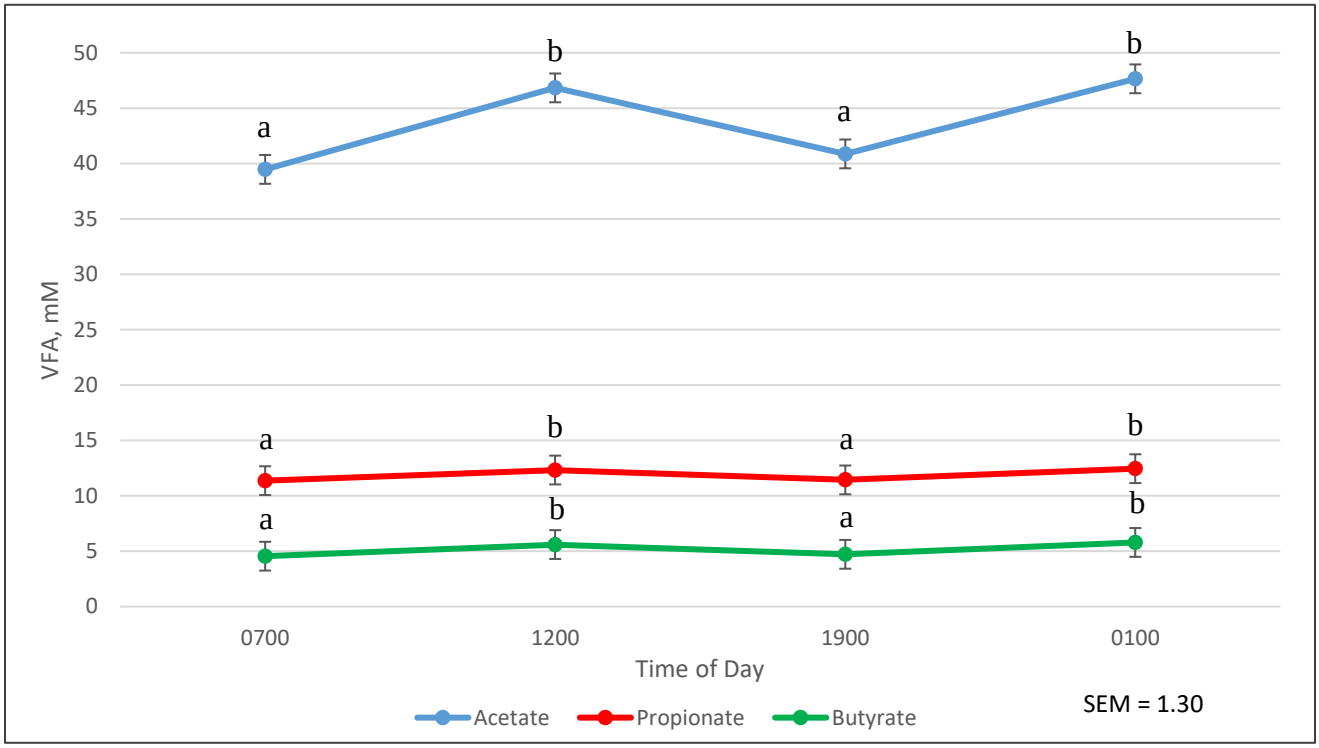


[†]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.5 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.

[a,b] Bars with different superscrpts differ ($P < 0.05$)

[‡]No treatment by time interaction ($P > 0.10$)

Figure 2.10 Effect of time on cecal acetate, proprionate, and butyrate concentrations[†,‡]



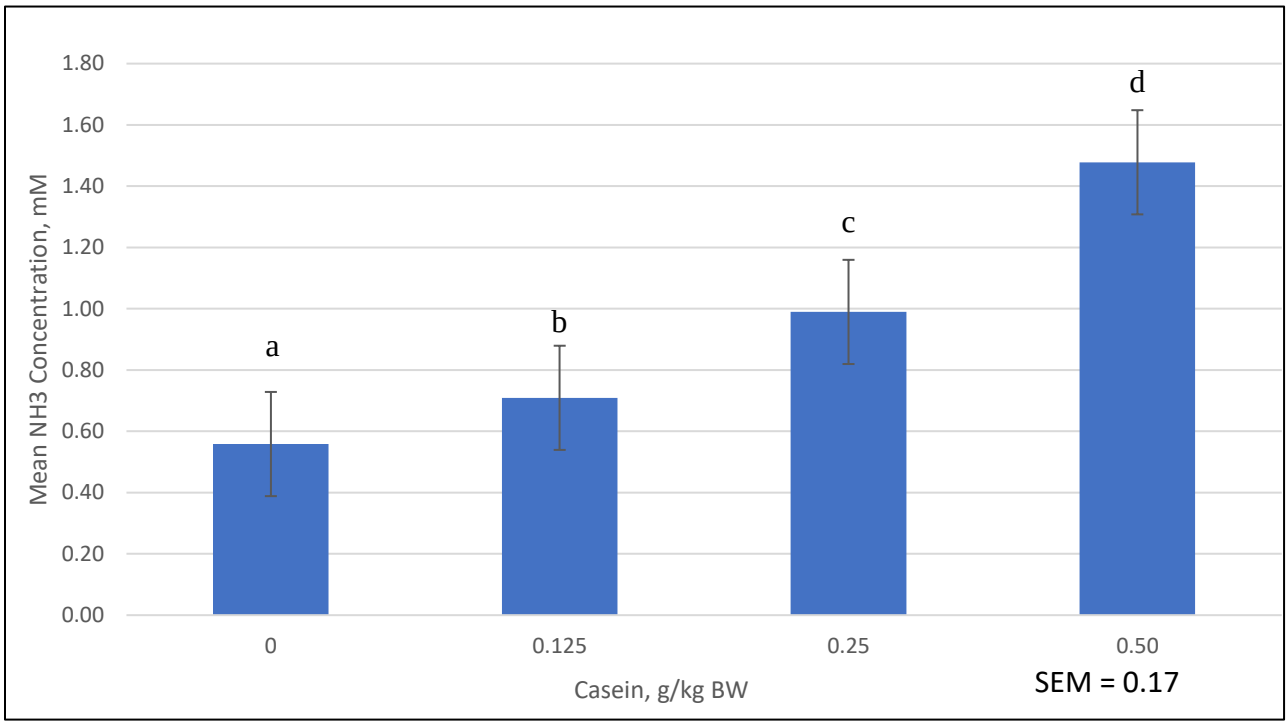
[†]Mean cecal VFA concentration across all treatment groups
[a,b]Means within a line with different superscript differ ($P < 0.01$)
[‡‡]No treatment by time interaction ($P > 0.10$)

Table 2.3 Apparent digestibility of Smooth Bromegrass hay

Treatment[†]	DM	OM	NDF	ADF
	…….%			
Control	46.30	46.26	44.78	45.47
Low	43.85	43.85	42.16	43.88
Medium	48.28	48.37	47.19	47.80
High	45.51	45.37	43.62	44.45
SEM	2.36	2.45	2.94	3.04
P-Value[††]	0.41	0.44	0.44	0.62

[†]Horses in the control group were administered 800 mL of distilled water via cecal cannulae. Horses in the low, medium, and high group were administered 0.125, 0.25, and 0.5 g/kg BW casein respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
[††]*P*-Value for overall model F-Test

Figure 2.11 Effect of cecally infused sodium caseinate on cecal NH^3 concentration[†,‡,‡‡]

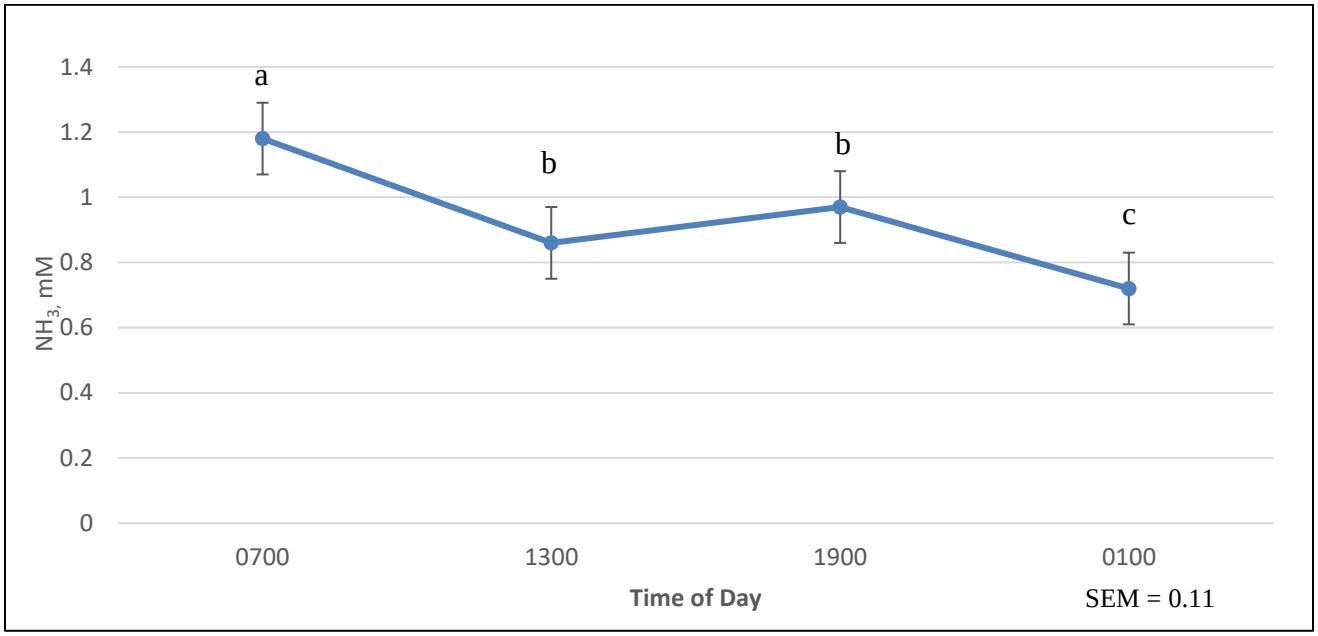


†Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.50 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.

[a,b,c,d]Values with different superscripts differ ($P < 0.01$)

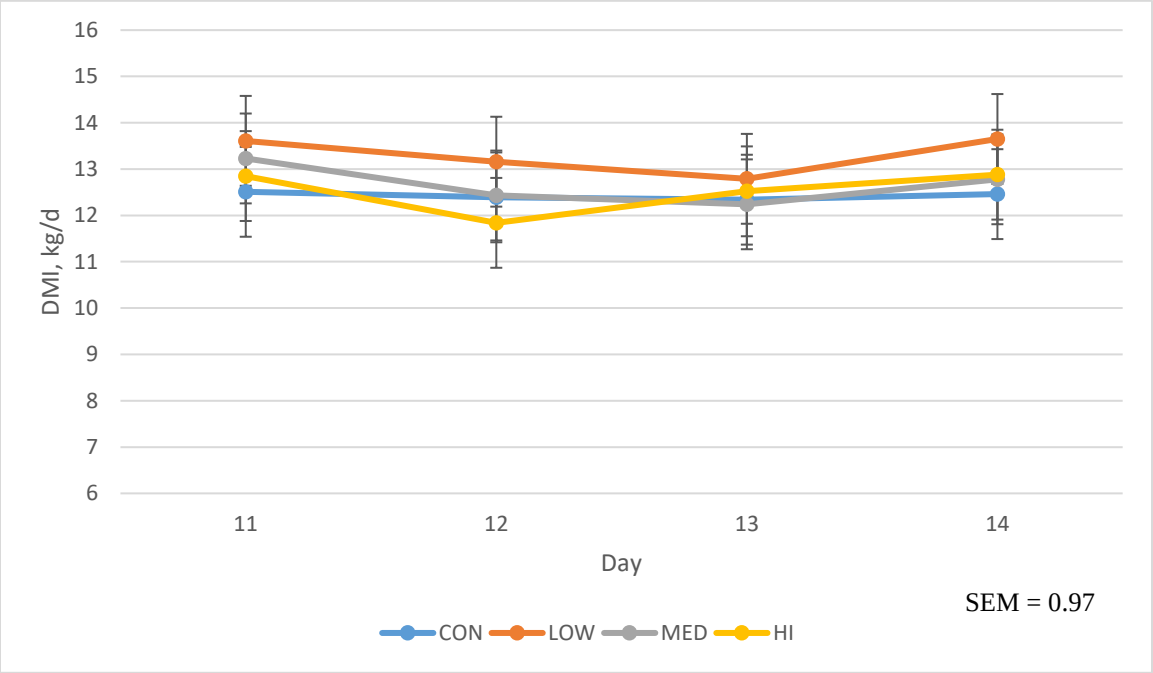
[‡]No treatment by time interaction ($P > 0.10$)

Figure 2.12 Effect of time on cecal NH_3 concentration[†,‡]



[†]Mean cecal NH_3 concentration across all treatment groups
[a,b,c]Values with different superscripts differ ($P < 0.05$)
[‡]No treatment by time interaction ($P > 0.10$)

Figure 2.13 Effect of cecally infused sodium caseinate on voluntary dry matter intake[†,‡,a,b,c]



[†]Mean DMI recorded on the last 4 d of each period. Horses were provided *ad libitum* Smooth Bromegrass hay and water. New hay was offered at 0700 and 1900.
[‡]Horses in the CON group were administered 800 mL of distilled water via cecal cannulae. Horses in the LOW, MED, and HI group were administered 0.125, 0.25, and 0.50 g/kg BW sodium caseinate respectively, solubilized in 800 mL of distilled water, via cecal cannulae.
[a]No effect of treatment ($P > 0.05$)
[b]No effect of day ($P > 0.05$)
[c]No day by treatment interaction ($P > 0.05$)

Chapter 3 - Literature Review:

Role of Probiotics and *Megasphaera elsdenii* in Domestic Livestock

Introduction

The use of antibiotics for growth promotion and to improve feed efficiency is no longer an option for livestock producers in the United States (US) after the Veterinary Feed Directive (VFD) became effective in January of 2017. This directive prohibits the use of "medically important" antibiotics for performance purposes. Producers can still use these drugs for their labeled purpose, but only through veterinarian-client-patient-relationship (FDA, 2015). The US is not the first country to implement a directive such as this. In 2006, the European Union (EU) banned the use of antimicrobial feed additives in response to concerns regarding antibiotic resistance. A subsequent decrease in feed efficiency and increase in disease prevalence occurred (Collins et al., 2009). Because of this, alternative strategies must be identified to improve performance and health of animals.

Among the species affected, poultry meat is the most common meat consumed in the US. In 2007, an estimated 8.6 billion broiler chickens sold in the U.S. compared to 96 million beef cattle (USDA, 2009). Ultimately, consumers play a large role in impacting the direction producers choose to go regarding the care and management of food animals. For example, the increased production of "naturally" raised animals and can be attributed to consumer demand and the fear of antibiotic resistance. This demand from consumers and implementation of the VFD together have encouraged the development of alternative means of maintaining animal

health while maximizing animal performance. One such option includes the use of probiotics to enhance digestive capacity and gastrointestinal health.

Poultry Microbiome

The microbiome that inhabits the gastrointestinal tract (GIT) of poultry is an integral partner in the symbiotic relationship between host and microbes. Through this relationship, the microflora utilize substrates and provide nutrients to the animal, while, in return, the animal provides a habitat in which microbes thrive. Microbes perform a number of functions that include fermentation of carbohydrates, vitamin synthesis, development and maintenance of intestinal morphology, detoxification, immunity response, and pathogenic defense (Chaucheyras-Durand and Durand, 2010; Pan and Yu, 2014). As a result, a healthy microfloral population is essential to animal performance and wellbeing (Pan and Yu, 2014). In fact, manipulation of microbial populations has enhanced performance through increased growth rates and decreased disease (Pedroso et al., 2013; Pan and Yu, 2014).

Before hatching, the GIT of the chick is considered sterile. Upon hatching, organisms that will colonize in the GIT are obtained from the egg shell and through maternal fecal matter. In modern agriculture, the chick and adult hen are separated to reduce the spread of disease. By decreasing exposure of the chick to fecal microbiota provided by the hen, the chick becomes increasingly reliant on environmental bacteria to populate its GIT (Pedroso et al., 2013).

The poultry GIT is much shorter than that of other mammals, and with an average rate of passage of 3.5 h, bacteria must grow rapidly or adhere to the mucosal layer to avoid elimination. Like the horse, the cecum of the chicken houses a complex ecosystem of bacteria. *Firmicutes, Bacteroidetes,* and *Proteobactera* were the most prevalent phyla represented in the intestine.

Within genera, *Clostridium*, *Ruminococcus, Lactobacillus*, and *Bacteroides* are most predominantly observed (Pan and Yu, 2014).

Probiotic Use in Poultry

The term probiotic is used to describe a live microbial organism fed to benefit the host through an improvement in microbial balance (Fuller, 1989). This term has been used loosely within the industry, being used to describe viable microbial cultures, enzymes, and cellular products. For this reason, the US Food and Drug Administration (FDA) now requires manufacturers to use the term "direct fed microbials" (DFM) to identify products containing naturally occurring microorganisms. Within the poultry industry, the most common probiotic strains used are members of the *Lactobacillus, Steptococcus, Bacillus, Enterococcus, Pediocuccus, Aspergillus*, or *Saccharomyces* genus (Dhama et al., 2011).

Because the GIT of the newborn chick is sterile after hatching, GIT colonization relies more heavily on environmental acquisition of microflora mainly due to the lack of contact from their mother and are therefore susceptible to infection in the 24 h of life. Opportunities for infection may arise if pathogenic bacteria are in their environment (Kabir et al., 2004; Chaucheyras-Durand and Durand, 2010). Supplementing DFM attenuates the effects of pathogens in young chicks. In one experiment, day old broiler chicks were dosed with a *Lactobacillus*-containing product and then challenged with *Salmonella enterica* and *Salmonella typhimurium*. Within 24 h, dosed birds had lesser cecal Salmonella populations when compared to the control (Higgins et al., 2007). Likewise, when *Lactobacillus* was administered to chicks challenged with *Campylobactor jejuni*, colonization of *C. jejuni* was reduced in the ceca (Neal-McKinney et al., 2012). La Ragione and Woodward (2003) demonstrated decreased shedding of

pathogens in broiler chickens dosed with *Bacillus subtilis* followed with a *Clostridium perfringens* and *S. enterica* challenge. Decreasing numbers of pathogenic bacteria is beneficial to both the bird and to humans in preventing pathogen transmission among birds in flocks and within the food system. Still others found no differences across treatments in pathogen prevalence in litter of broilers supplemented with a variety of commercially available probiotic products (Pedroso et al., 2013).

Supplementing probiotics has been shown to improve layer performance, as measured through several parameters. Improvements in egg production, egg weight, and specific gravity has been seen in layer hens supplemented with *Lactobacillus* (Tortuero and Fernandez, 1995; Haddadin et al., 1996; Nahashon et al., 1996). However, others found no difference in layer performance when supplemented with a probiotic (Mohan et al., 1995; Chen and Chen, 2003).

Mode of Action

The efficacy of DFM in improving animal health and performance can be attributed to several modes of action, including increased nutrient digestibility and absorption, competitive exclusion of pathogenic microbes, production of antibacterial compounds, and immunomodulation (Dhama et al., 2011). The mode of action of DFM have become increasingly studied among a wide variety of species to include horses, cattle, poultry, dogs, and humans.

The most commonly reported mode of action whereby probiotics operate to improve health and performance is though competitive exclusion or bacterial antagonism (Mack et al., 1999). By competing for nutrients, probiotics decrease the substrate for pathogenic bacteria, consequently inhibiting their growth and function. Production of bacteriocins can cause death of pathogenic bacteria and interfere with colonization. These antimicrobial molecules have been shown to inhibit the binding of *Salmonella* and *Shigella* to intestinal epithelial cells extracted

from the chicken (Gusils et al., 2003). Production of bacteriocins can inhibit the activity of closely related bacteria. One such example is the production of enterocin from *Enterococcus faecium*. This bacteriocin inhibits growth of several pathogens, including *E. Coli,* listeria, enterococci, and staphylococci (Laukova et al., 1998). Production of VFA, organic acids, and lactic acids by DFM can modify the intestinal tract, thus lowering pH and creating an unfavorable environment for pathogenic bacteria like *E. Coli* and *Salmonella* (Dhama et al., 2011). Neal-McKinney et al. (2012) found that production of lactic acid from Lactobacillus disrupted the membrane of *C. jejuni* when studied *in vitro*. Additionally, death of *C. jejuni* was observed 6 h quicker in media containing 25 mM lactic acid versus media contacting HCl at the equivalent pH (Neal-McKinney et al., 2012).

Direct fed microbials have also been reported to result in immunostimulation which is thought to be accomplished through increased lymphocytes and lymphoid cells in lamina propria and its intra-ephithelial lymphocytes (Dhama et al., 2011). Increased natural T-killer cells and phagocytic activity of leukocytes have been reported in humans and animals supplemented with DFM (Fuller, 1989; Gill, 2001). Primalec, a DFM containing *Lactobacillus acidophilus, Lactobacillus rhamnosus, Enterococcus faecium,* and *Bifidobacterium thermophilum,* has been shown to enhance antibody production when provided to broiler chickens (Nayebpor et al., 2007). Morevover, immunomodulation was demonstrated in chickens orally dosed with *Lactobacillus* and was characterized by enhanced serum IgM and IgG (Koenen et al., 2004).

More commonly, probiotics have been shown to enhance nutrient digestion and utilization resulting in improved ADG and feed efficiency (FE). Yeasts have been used in horses and cattle to increase dry matter digestibility and substrate utilization. Acid detergent fiber digestibility was increased in horses supplemented with *Sacchromyces cerevisiae* (SC) that were

fed a high fat or high starch diet (Jouany et al., 2008). Researchers later determined that this increase in ADF digestibility was due to promotion of fibrolytic enzymatic activity (Jouany et al., 2009). However, Lattimer et al. (2007) found no differences in digestibility or fermentation when using an *in vitro* model to compare equine fecal cultures with or without SC. When supplemented to dairy cattle, *S. cerevisiae* improved protein and cellulose digestion resulting in a greater DMI (Wohlt et al., 1991). Several authors reported stimulation in growth and increased feed efficiency with DFM supplementation in birds (Mohan et al., 1996; Lan et al., 2003; Khan et al., 2007). However, other reports have been inconsistent with these findings. Gunal et al. (2006) indicated no changes in weight gain, feed intake, or feed conversion ratio in broilers supplemented with an antibiotic, probiotic, or organic acid. Similar reports of unaffected performance in supplemented birds were reported by Panda et al. (2000) and Watkins and Kratzer (1984). Environmental conditions may play an important role, as Anderson et al. (1999) found no differences in performance among birds in clean conditions and maintained at a proper stocking density when treated with a variety of growth promoters.

Megasphaera elsdenii

Formerly known as *Peptostreptococcus elsdenii, M. elsdenii* was first isolated and identified in the rumen of sheep (Lewis and Elsden, 1955). A similar strain (KS-249) has been identified in the cecum of a horse consuming a high forage diet (Leventhal et al., 2011). Upon 16S rRNA sequencing, strains isolated from a lamb, calf, and sheep all appeared to have similar genetic make-up (Piknová et al., 2006). Moreover, it was concluded that even with variances in environment (South Africa versus the US), there is limited genetic variability among strains (Piknová et al., 2006).

Although *M. elsdenii* is a naturally occurring bacterium, its use as a probiotic in poultry has been negligible. Previous researchers have primarily investigated its use in reducing the prevalence of acidosis in the ruminant and equine. This gram-negative coccus reportedly ferments up to 97% of ruminal lactate, making it the predominant LUB within the rumen (Counotte et al., 1981). However, *M. elsdenii* can effectively metabolize a range of carbon sources. Specifically, *M. elsdenii* strain 41125 has been shown to utilize fructose, glucose, lactate, and maltose as alternative substrates (Mobglia et al., 2016). *Megasphaera elsdenii* is unique from other LUB in that it is viable in pH ranging from 4.7 to 7.8 while also being more oxygen tolerant (Lewis and Elsden, 1955; Therion et al., 1982 ; Meisser et al., 2010).

Megasphaera elsdenii converts lactate to propionate using the acrylate pathway rather that the succinate pathway (Fig 3.1). Upon fermentation, lactate is converted to propionate 5 times fasters than glucose (Hino et al., 1994). Propionate provides the animal a major energy source (Hino et al., 1994). In addition, *M. elsdenii* can produce a significant amount of butyrate. Buyrate is integral in epithelial growth and mucosal health. In the rumen, butyrate is largely metabolized in the epithelium and is propionate sparing (Van Soest, 1994; Baldwin and McLeod; 2000). When dosed with *M. elsdenii*, large intestine muscosa of rats was thicker and greater epithelial cell numbers were observed than those that did not receive the DFM, which was attributed to greater butyrate production (Hashizume et al., 2003).

Allison (1969) theorized that *Megasphaera elsdenii* plays an integral role in the production of branched chain VFA in the rumen (Allison, 1969). The proteolytic function of *M. elsdenii* was discovered *in vitro* when increased amounts of isobutyrate, isovalerate, and valerate were associated with decreases in lactate accumulation. Through deamination of branched chain

AA, *M. elsdenii* provides branched chain fatty acids are readily available for fiber digestion (Kung and Hession, 1995).

Many researchers have investigated the role of *M. elsdenii* in transitioning feedlot cattle to high grain diets. Rapid transition to a high concentrate diet increases the risk of ruminal acidosis, lactic acid accumulation, and subsequently declining in pH. Although *M. elsdenii* is a naturally occurring LUB in the rumen, supplementation of *M. elsdenii* effectively eases the transition to a high concentrate diet though enhanced lactate utilization (Drouillard et al., 2012). Isolated from concentrate-fed cattle, *M. elsdenii* strain NCIMB 41125 has been shown to have an accelerated growth rate, tolerance to low pH, and is not affected by ionophore antibiotics (Marounek et al.,1989; Callaway et al., 1999; Henning et al., 2010). More recently, Meissner and others (2014) reported that *M. elsdenii* strain 41125 was unaffected by non-ionophore products *in vitro*. Furthermore, increases in total VFA and propionate production were observed in cattle when *M. elsdenii* NCIMB 41125 was used in conjunction with zinc bacitracin or tylosin (Meissner et al., 2014). With supplementation, producers may have the ability to decrease length of step-up programs in feedlot cattle, thus reducing cost of production.

Megasphaera elsdenii as a DFM in Poultry

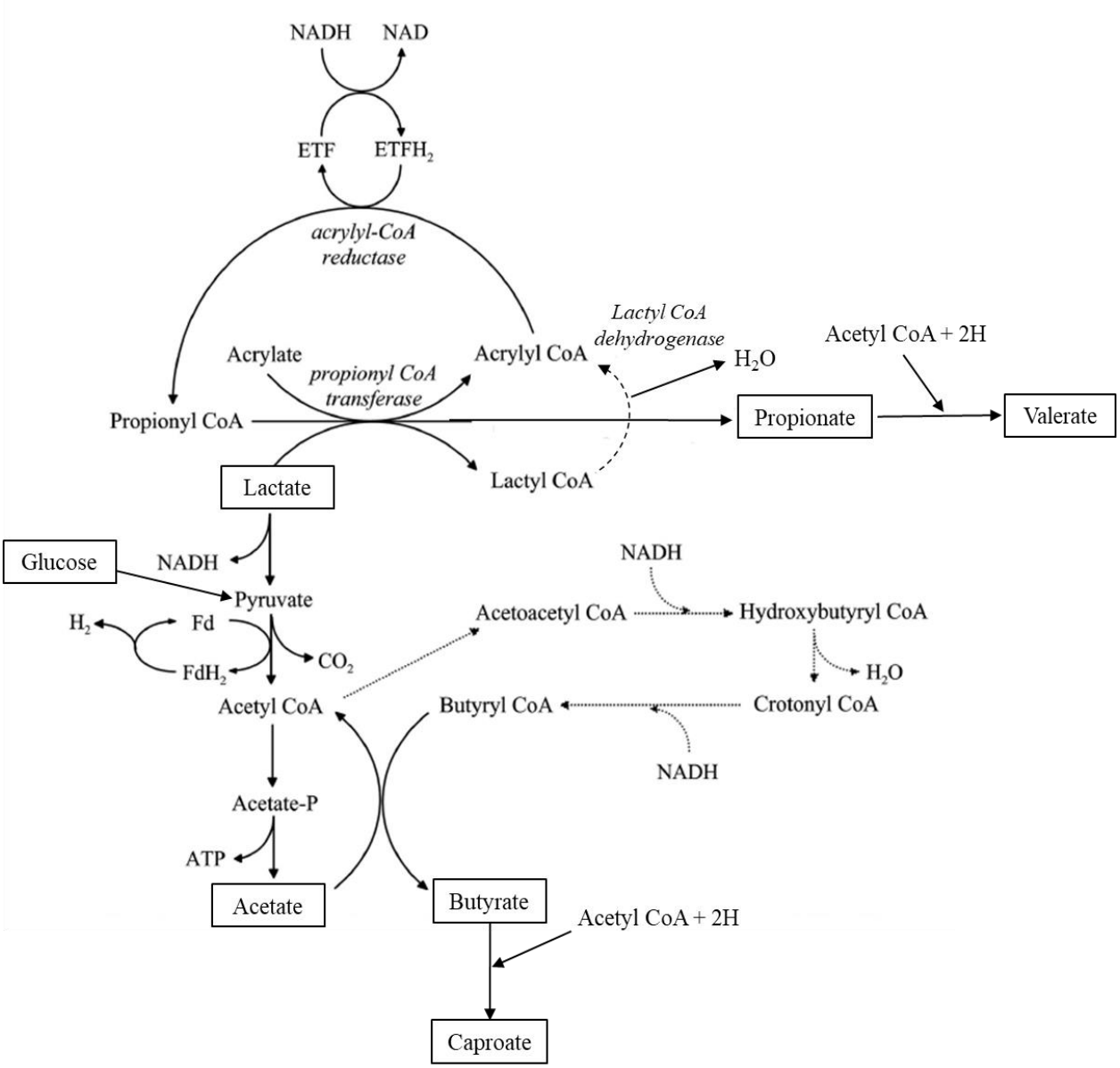
The reported usage of *M. elsdenii* as a DFM in broiler chickens has been limited. When provided as an oral gavage or lyophilized feed additive, FE increased 4.6% during the first 21 d of life (Drouillard, person communication). In another study, increased FE in chickens supplemented with *M. elsdenii* in the form of an aerosolized mist or oral gavage was observed (Drouillard, personal communication). In a third unpublished study, oral and misted supplementation had no effect on feed intake, FE, nor ADG (Drouillard, personal communication). Researchers speculated that birds may have cross-contaminated each other,

thus *M. elsdenii* might have colonized in the GIT of control birds. In the same study, cecal pH was lesser in birds treated with *M. elsdenii* thus researchers suggested that it may be advantageous to use as an acidifier. Acidifiers are commonly used in the poultry industry to decrease the prevalence of pathogenic organisms (Aclkgoz et al., 2011).

Summary

With the implementation of the VFD, the need to find alternatives to antibiotics for growth promotion and increased production has become a priority. Probiotics or DFM have been shown to be effective at promoting growth and health status in poultry (Kabir et al., 2004; Khan et al., 2007; Dersjant-Li et al., 2016). *M. elsdenii,* has primarily been used in ruminants to attenuate the effects of ruminal acidosis through enhancement of the resident microbial population. However, a small number of research trials conducted in broiler chicks have demonstrated the potential for the use of *M. elsdenii* as a DFM in poultry through improvement of FE and ADG. Therefore, this study was performed to evaluate the effects of strain and application method of *M. elsdenii* on broiler chick growth performance.

Figure 3.1 Proposed pathway for synthesis of propionate, butyrate, valerate, and caproate from lactate or acrylate by *Megasphaera elsdenii* (adapted from Prabhu et al., 2012 and Weimer and Moen, 2013)



Chapter 4 - Effect of *Megasphaera elsdenii* on growth performance of broiler chicks

K.V. Jordan, J.S. Drouillard, T.J. Ellerman, T.L. Douthit, J.M. Lattimer

Kansas State University

Manhattan, KS 66506-1600

Abstract

Two battery studies were conducted to evaluate the effect of application method and strain of *M. elsdenii* supplementation on growth performance of broiler chickens. For both experiments, day-old male Cobb 500 broiler chicks blocked by cage location and treatments were randomly assigned to pens within each block. Chicks were housed in battery cages equipped with raised wire mesh floors. Feed and water were provided *ad libitum*. Feed intake and pen weight data were collected on D 1 and D 18 after ending the study. In Experiment 1, pens were randomly assigned to 1 of 6 treatments: 0.2 mL oral gavage containing 1.97 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 (MS-Biotec, Wamego, KS; O-L), 0.2 mL of fresh culture containing 0 CFU/mL of *M. elsdenii* strain KS 249 (O-KS; Attempts to grow this strain were unsuccessful), 0.2 mL of a fresh culture containing 1.06 x 10^9 CFU/mL of *M. elsdenii* strain B52-2083 (O-B52), aerosolized mist at rate of 15 mL per pen containing 1.97 x109 CFU/mL of *M. elsdenii* strain NCIMB 41125 (~1.88 mL/bird; MS-Biotec, Wamego, KS; OM), topdressing (mixture of diet and freeze dried *M. elsdenii* strain NCIMB 41125; TD) containing 1.18 x 10^7 CFU/g of *M. elsdenii* strain NCIMB 41125 at a rate of a quarter teaspoon per bird, or negative control that had no contact with the probiotic product. Feed intake was similar across all treatments ($P = 0.82$). Similarly, there was no effect of treatment on ADG ($P = 0.89$), gain:feed ($P = 0.93$), nor mortality ($P = 0.54$). In Experiment 2, chicks were assigned to 1 of 2 treatments consisting of lyophilized Lactipro in the form of a topdressing (TD) or a negative control that had no contact with the probiotic product. Average daily gain ($P = 0.02$) and gain:feed ($P = 0.04$) were both greater in birds receiving the TD when compared to control. Feed intake ($P = 0.70$) and mortality ($P = 0.31$) were not different among treatments. Because of the increased in ADG

and FE, administration of lyophilized *M. elsdenii* strain NCIMB 41125 may be an effective means to enhance growth performance of broiler chicks.

Introduction

The cecal microflora in broiler chickens is critical for maintaining proper gut function and health. Enhancement of the microbiome and prevention of dysbiosis has the potential to improve growth performance. Antimicrobial compounds have been used to achieve these goals by manipulating select populations of microbes that colonize the gastrointestinal tract. As of January 1, 2017, the use of medically important antibiotics as growth promotants has been abolished. Both the U.S. and E.U. have banned the sub-therapeutic usage of antibiotics due to rising concerns of antibiotic resistance, which, in turn, has challenged farmers to use new husbandry practices. Probiotics, exogenous enzymes, acidifiers, and select minerals have all been explored as alternatives to antibiotic usage.

Among the possible alternatives, probiotics have been increasingly investigated. The addition of live microorganisms improves animal productivity and health through reduction of pathogens and immunomodulation. There is increasing evidence that these objectives may be accomplished through the use through a variety of gram-negative bacteria in poultry (Balevi et al., 2001; Huang et al., 2006; Neal-McKinney et al., 2012).

Megasphaera elsdenii, a relatively unexplored option, naturally colonizes the lower gastrointestinal tract of mammals and birds (Scupham et al., 2003). Under modern agricultural practices, its numbers are relatively low in the GI tract of most farm animals. *M. elsdenii* has been used in cattle to attenuate the effects of ruminal acidosis though its ability to ferment large quantities of lactate; however, based on a small number of studies performed in broiler chicks at Kansas State University, researchers have implicated the potential use of *M. elsdeni* as a DFM in poultry (Drouillard, personal communication. The purpose of this study was to determine the effects of strain and application method of *M. elsdenii* on growth performance in broiler chicks.

Materials and Methods

Animals and Housing

All procedures related to the care and handling of animals were approved by the Kansas State University Institutional Animal Care and Use Committee. Day-old male Cobb 500 broiler chicks with an average BW of 41.83±2.15 g were obtained from Cobb-Vantress in Siloam Springs, Arkansas, and transported to the Kansas State University Poultry Research Center. Chicks were housed in battery cages equipped with raised wire mesh floors and located within a single feeding barn. Each battery consisted of 6 tiers with 4 pens in each tier.

Experiment 1

This experiment was conducted as a randomized complete block design with 18 replicates of 6 treatments which were blocked by battery and tier to account for ventilation and temperature differences. Experimental unit was pen (n = 108), and each pen contained 8 birds at initiation of the experiment (1,152 total birds). Groups of birds were processed as blocks, and experimental treatments were assigned randomly to pens within each block.

Three different strains of *M. elsdenii* were used. The strain KS-249, previously isolated in our lab from the cecum of a horse, was administered as an oral gavage (Douthit, personal communication). Lactipro, a commercially available product used in cattle containing the strain NCIMB 41125, was utilized in 3 manners: 1) an aerosolized mist applied to the body 2) an oral gavage 3) a lyophilized culture mixed in a corn-soy diet to form a top dressing (MS-Biotec, Wamego, KS). The B52 strain was only administered as an oral gavage.

Treatments consisted of the following: 0.2 mL oral dose of *M. elsdenii* containing 1.97 x 10^9 CFU/mL strain NCIMB 41125 (Lactipro, MS-Biotec, Wamego, KS); 0.2 mL oral dose of a fresh culture containing 0 x 10^9 CFU/mL of *M. elsdenii* strain KS 249 (No counts were able to be verified at this dilution); 0.2 mL oral dose of a fresh culture containing 1.06 x 10^9 CFU/mL of *M. elsdenii* strain B52-2083; topical mist of a fresh culture containing 1.97 x10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 (~1.88 mL/bird; MS-Biotec, Wamego, KS); freeze dried topdress containing 1.09 x 10^7 CFU/g of *M. elsdenii* strain NCIMB 41125 (MS-Biotec, Wamego, KS) at a rate of a 0.925 g per bird; control which had no contact with the DFM. No counts of the KS 249 strain could be verified at 10^5 nor 10^6 dilutions, however there may have been growth at lower dilutions.

Birds dosed with oral treatments were restrained in the palm of a technician's hand, the beak was held open using the thumb and forefinger, and culture was discharged directly into the oral cavity using an Eppendorf Reference repeater pipette (Eppendorf, Hamburg, Germany). Birds dosed with the topical mist were placed in a plastic tub (50 cm long x 35 cm wide, x 40 cm deep) and culture was applied to the body surface of birds as an atomized mist using a pneumatic drenching device fitted with an atomizing tip. Pens that were assigned to the misted treatment were misted once upon initiation of the study and treatment was applied to each bird in the pen at the same time. Misted birds were handled by designated personnel and placed in designated carriers for weighing, application and transfer to pens to minimize cross-contamination. The topdress treatment was added directly into the trough feeders daily at 1300 h starting on d 10 of the study.

Fresh water was available *ad libitum*. Prior to placing birds in pens, 9.5 kg of a common starter diet (Fig 4.1) were placed into trough feeders alongside each pen. Feed was replenished

as needed to ensure *ad libitum* access throughout the study. Upon termination (d 18) of the experiment, unconsumed feed was removed from each feeder, weighed, and recorded. Total feed consumed by the pen was calculated as the difference between amounts added to and recovered from feeders. Daily feed intake per bird was calculated as:

total feed consumed ÷ [daily head count in pen x total days on feed]

At both the start and end of the study, all birds in each pen were placed into a tub (50 cm long x 35 cm wide, x 40 cm deep) and weighed. Initial tub weight was subtracted from the total weight with birds to ascertain weight of the birds in the pen. Head count verification was also performed at this time. Average daily gain was calculated as:

$$\frac{[initial\ pen\ weight - ending\ pen\ weight]}{[daily\ head\ count\ in\ pen\ x\ total\ days\ on\ feed]}$$

Statistical Analyses

Data were analyzed using the Mixed procedure of SAS 9.4. The model included fixed effect of treatment, random effect of block, and pen as the experimental unit. Treatments were blocked by battery and by tier. Significance was declared at $P < 0.05$. A tendency was considered to be $0.05 < P \leq 0.10$. Differences among least squares means were determined using the PDiff option of SAS.

Experiment 2

This experiment was conducted as a randomized complete block design with 18 replicates of 2 treatments, blocked by battery and tier. Chicks were housed in battery cages equipped with raised wire mesh floors and located within a single feeding barn. Each battery consisted of 6 tiers with 4 pens in each tier. In order to account for differences in ventilation and

temperature, battery and tier were used as a blocking criterion. Treatments were randomly assigned to pens within each block. Birds were processed as blocks, and experimental treatments were assigned randomly to pens within each block.

Treatments consisted of the following: lyophilized topdress containing 1.18 x 10^7 CFU/g of *Megasphaera elsdenii* strain NCIMB 41125 at a rate of a 0.925 g per bird; control which and had no contact with the DFM. Control birds were handled by designated personnel and placed in designated carriers for weighing and transferring to pens to minimize cross-contamination by treated birds. Administration of feed and water and the weighing of birds were as described in Exp. 1.

Statistical Analyses

Data were analyzed using the Mixed procedure of SAS 9.4. The model included fixed effect of treatment, random effect of block, and pen as the experimental unit. Significance was declared at $P < 0.05$. A tendency was considered to be $0.05 < P \leq 0.10$. Differences among least squares means were determined using the PDiff option of SAS.

Results

Experiment 1

Broilers across all treatment groups showed similar daily feed intake, ADG, gain:feed, and mortality (Table 4.2). Daily feed intake values ranged from 36.4 to 37.7 g/d ($P = 0.82$) among treatments. Chicks across all treatments had comparable average daily gains, ranging from 27.9 to 28.8 g across all treatments ($P = 0.89$). Furthermore, gain:feed means were similar for all treatment groups, ranging from 0.75 and 0.77 ($P = 0.93$). There were no mortalities in

birds orally dosed with the KS-249 strain, while mortalities for the remainder of the treatments ranged from 1.39 to 2.22% ($P = 0.54$).

Experiment 2

Average daily gain and gain:feed were both greater in birds receiving lyophilized *M. elsdenii* when compared to control birds (Table 4.3). Birds in the topdress group had an ADG of 29 g compared control birds who had an ADG of 27.60 g ($P = 0.02$). There was a 5% increase in gain:feed, from 0.76 to 0.80, in the topdress group compared to the control birds ($P = 0.04$).

Feed intake and mortality in the control and topdress group were not different between treatments. When on the control and topdress treatments, birds consumed an average of 36.20 and 36.50 g, respectively, of the diet per d ($P = 0.70$). Mean mortality percentages were 2.08 and 0.69 for the control and topdress groups, respectively ($P = 0.31$).

Discussion

Neither strain nor application method of *M. elsdenii* impacted growth performance of broiler chicks in Exp. 1. This contradicts findings of Exp. 2, as well as 2 previous studies conducted at Kansas State University (Drouillard, person communication). Furthermore, others have reported enhanced growth performance through probiotic supplementation in broiler chickens and layer hens (Kabir et al., 2004; Khan et al., 2007; Dhama et al., 2011). Many authors suggest that probiotics are most effective under stressful conditions such as initial processing, temperature variation, overcrowding, or disease challenge (Patterson and Burkholder, 2003; Kabir et al., 2004). Because the current experimental conditions did not present any unusual stressors, all birds may have experienced similar growths and low mortalities, regardless of treatments.

Cross-contamination should be considered after finding no performance differences in Exp. 1. Although precautions were taken to prevent cross-contamination, treatment groups were not completely isolated from one another. It is possible that control birds may have consumed the bacterium ensuing colonization. However, cecal contents would need to be analyzed to confirm or dismiss this theory.

Daily administration of this product provided benefits to growth of birds in Exp. 2. Administration of lyophilized *M. elsdenii* improved both the ADG and FE of broiler chicks compared to non-supplemented birds. Daily administration may be necessary due to the relatively rapid rate of passage in chicks. This improvement in animal performance may be a result of improved nutrient digestion or absorptive capacity. However, it is unclear as to why findings of 2 experiments disagree as to the efficacy of lyophilized *M. elsdenii*. The increase in performance observed in Exp. 2 implies that *M. elsdenii* was utilized within the GIT. Further studies should examine cecal contents to observe any changes occurring within the microbiome. In both studies, mortalities were no different across treatments, verifying that *M. elsdenii* is not detrimental to broiler health.

Conclusion

M. elsdenii has neither a detrimental nor beneficial effect in broiler chick performance in Exp. 1. However, results of Exp. 2 contradict this data, illustrating improvements in ADG and FE with daily administration of lyophilized *M. eldsdenii*. Further research should focus on the efficacy and viability of lyophilized strains of *M. elsdenii.* Although an anaerobic bacterium, a mode whereby *M. elsdenii* was readily available to be mixed into feed, would prove to be valuable.

Limitations

A significant amount time was spent dosing chicks via oral gavage. Deemed acceptable for a research trial and smaller farms, this would prove to be impractical in commercial poultry production. While this method allows technicians to confirm ingestion of the bacterium, extended handling may add un-needed stress to an already taxing situation for the chick. Attempts to plate *M. elsdenii* KS-249 strain at dilutions 10^5 and 10^6 were unsuccessful upon completion of dosing on d 1. This may indicate that no live cultures were administered to chicks assigned to this treatment group. Another limitation of this study was the delayed supplementation of the topdress. Lyophilized *M. elsdenii* was not added to the diet until d 10 of the study due to delays in efficacy of freeze-drying process. Although there were differences among treatments, an even larger difference might have been observed had supplementation started on d 1.

Table 4.1 Composition of broiler diet[†]

Ingredient, %	
Ground corn	55.26
Dehulled soybean mean, 47% CP	37.15
Soybean oil	3.10
Ground limestone	1.45
Salt	0.37
Monocalcium phosphate, 21%[*]	1.70
Sodium bicarbonate	0.22
Vitamin and mineral premix[ǂ]	0.25
L-lysine hydrochloride	0.33
L-methionine	0.13
L-threonine	0.04

[†]Diets were pelleted through a 3-mm diet, cooled, crumbled. Diet was provided *ad libitum*.
*Biofos®, Mosaic Co., Plymouth, MN
[ǂ]Nutrablend poulty VTM premix, Neosho, MO

Table 4.2 Effect of *Megasphaera elsdenii* on broiler performance in Experiment 1

		Oral gavage[2]			Aerosol[3]	Topdressing[4]		
Item	**Control[1]**	**NCIMB 41125**	**B52-2083**	**KS-249**	**NCIMB 41125**	**NCIMB 41125**	**SEM**	***P*-Value[5]**
No. of pens	18	18	18	18	18	18	-	-
ADG, g	28.5	28.8	28.2	28.2	27.9	28.1	0.61	0.89
Feed intake, g	37.3	37.7	37.5	37.2	36.4	37.1	0.76	0.82
Gain:feed	0.77	0.76	0.75	0.76	0.77	0.76	0.01	0.93
Mortalities, %	2.22	1.39	2.08	0	2.33	1.39	1.08	0.54

[1]Control treatment, no probiotic

[2]*Megasphaera elsdenii* cultures were administered directly to the oral cavity at a rate of 0.2 mL/bird using an Eppendorf Reference repeater pipette (Hamburg, Germany)

[3]Aerosolized *Megasphaera elsdenii* strain NCIMB 41125 was applied directly to the body surface at a rate of ~1.88 mL/bird using a drenching device equipped with an atomizing tip (1.97×10^9 CFU/mL).

[4]Lyophilized *Megasphaera elsdenii* strain NCIMB 41125 was administered daily in the form of a topdressing at a rate of 0.925 g per bird. (1.18×10^7 CFU/g).

[5]P-value for overall model F-test

Table 4.3 Effect of *Megasphaera elsdenii* on broiler growth performance in Experiment 2

Item	Control [1]	NCIMB 41125[2]	SEM	*P*-Value [3]
No. of pens	18	18	-	-
ADG, g	27.60[a]	29.0[b]	0.43	0.02
Feed intake, g/d	36.20	36.50	0.54	0.70
Gain:feed	0.76 [a]	0.80 [b]	0.01	0.04
Mortalities, %	2.08	0.69	0.93	0.31

[1]Control treatment, no probiotic

[2]Lyophilized *Megasphaera elsdenii* strain NCIMB 41125 was administered daily in the form of a topdressing at a rate of 0.925 g per bird (1.97×10^9 CFU/g).

[3]P-value for overall model F-test

[a, b] Means within a row with different superscripts are different at $P < 0.05$

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