Use of *Megasphaera elsdenii* strain NCIMB 41125 as a direct fed microbial in monogastric livestock and poultry

by

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Abstract

Three experiments were conducted to determine the impact of *Megasphaera elsdenii* supplementation in monogastric livestock and poultry. Experiment 1 evaluated effects of *M. elsdenii* on growth performance and cecal parameters in broiler chickens (n = 2520). Birds in treatment groups 1 and 2 were administered *M. elsdenii* culture as either an oral gavage (OG) or an aerosolized mist (AM), respectively. Treatment group 3 served as a negative control (C). Growth performance and feed efficiency (FE) were unaffected by treatment ($P > 0.10$). Cecal pH was reduced in AM and OG birds compared to C ($P < 0.05$). *M. elsdenii* is most effective in altering cecal VFA profile before d 21 post-administration, after which no differences in VFA concentrations were detected between treatments. Reduction in cecal pH in response to *M. elsdenii* indicates potential application of this probiotic as an acidifier, commonly used in poultry production as an antibiotic alternative. Experiment 2 was a swine performance study conducted to determine effects of treating sows or piglets or both with an oral gavage of *M. elsdenii*. Factor 1 consisted of administration of an *M. elsdenii* oral gavage administered to sows or a negative control (n = 28). Factor 2 consisted of either a negative control or oral gavage of *M. elsdenii* administered to litters of piglets shortly after birth and at weaning. Sow weight loss during lactation was unaffected by treatment ($P > 0.10$). Feed intake upon introduction to the nursery was greater in piglets that received *M. elsdenii* directly than those that did not ($P < 0.05$). Large intestinal fermentation in both sows and piglets was affected by *M. elsdenii* administration. Fecal VFA concentrations tended to be greater in animals that received *M. elsdenii* either indirectly through the sow or directly via oral gavage ($P < 0.05$). Direct supplementation of piglets with *M. elsdenii* is more effective in altering fermentation after d 21 than indirect administration, as VFA concentrations in piglets that received *M. elsdenii* indirectly are similar to the control by d 28.

Butyrate concentrations were greater in piglets that received *M. elsdenii*; this VFA is important in prevention of intestinal atrophy and recovery from physiological stress of weaning. Experiment 3 examined effects of *M. elsdenii* administered as either an oral drench (OD) or a lyophilized powder (LP) on equine cecal fermentation compared to a negative control (NC). Cecally cannulated horses (n = 8) were transitioned to a 1:1 roughage to concentrate diet over a period of 5 d. Cecal pH was greater than NC in OD and LP horses on d 5 and d 7, respectively ($P < 0.10$). Acetate:propionate ratio, valerate, and caproate concentrations also were greater in LP horses than NC on d 7 ($P < 0.05$). This may indicate improved gut health and fiber digestion in supplemented horses. *M. elsdenii* is able to effectively alter large intestinal fermentation in monogastric species and may have potential to improve gut health and performance of these species.

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“Lord you establish peace for us, everything we have accomplished you have done for us.”

Isaiah 26:12

Dedication

I dedicate this thesis to both of my grandfathers, Joe Scaletti and Ray Romatowski. You inspired in me a love of science at an early age and nurtured that love as I grew. I am honored to have followed in your footsteps in my education and I hope I have made you both proud.

Chapter 1 - A Review of the Literature

1.1 Introduction

Probiotic use in livestock production as a possible alternative to antimicrobial feed additives has gained considerable interest in recent years. Regular use of antibiotics for growth promotion and maintenance of animal health status is rapidly falling out of favor (Cromwell, 2001; cited in Collins et al., 2009). This is largely due to growing public concern over the potential for antibiotic resistance and subsequent ban of antimicrobial feed additives by the European Union (EU) in 2006. This ban has led to increased disease and decreased feed efficiency, causing significant economic impact (Collins et al., 2009). Therefore, an alternative strategy to improve animal health and performance while minimizing environmental impact is essential (Yirga, 2015). This need will continue to grow with the introduction of the Veterinary Feed Directive (VFD) in the United States in January of 2017. The directive will disallow the use of "medically important" antibiotics for growth promotion and limit their therapeutic use without the supervision of a veterinarian (FDA, 2015). Probiotic feed additives are an attractive "natural" substitute to antibiotics to modulate gastrointestinal (GI) health, improve animal productivity, and reduce pathogenic infection (Yirga, 2015; Chaucheyras-Durand and Durand, 2010).

1.2 Establishment of the gastrointestinal tract's microbiome

The gastrointestinal tract (GIT) is home to large, diverse communities of microorganisms including bacteria, fungi and protozoa (Savage, 1977; Mackie et al., 1999). The primary functions of these microorganisms are the breakdown and fermentation of carbohydrates, endogenous vitamin synthesis, detoxification of toxic compounds, immunostimulation, and maintenance of gut integrity to prevent pathogen infection (Chaucheyras-Durand and Durand, 2010). Due to these important roles in the gastrointestinal tract, status of the microbial ecology

within the gut will directly direct impact the health and productivity of the host (Mackie et al., 1999; Agarwal et al., 2002).

In utero, the GIT is considered mostly sterile. Microorganisms are introduced to the digestive system during parturition and shortly thereafter by the dam and environment. During the first few days of life, the microbiome of the neonate begins to develop into a complex ecosystem which will remain in transition as the animal matures and begins to consume solid food (Savage, 1977; Mackie et al., 1999). Level, order and type of microbial colonization in the neonate are heavily influenced by environmental and dietary exposure in the peripartum period (Mackie et al., 1999). Most commonly, colonization begins with lactate-producing bacterial (LAB) species such as streptococci and enterobacteria while the neonate is nursing. Following weaning and the introduction of solid food, the number of facultative anaerobic bacteria decrease while populations of obligate anaerobic bacteria increase in number and complexity until stabilizing into a pattern that will remain with little variation into adulthood (Savage, 1977; Mackie et al., 1999; Konstantinov et al., 2004).

Most of the microbial population of the GIT of monogastrics is contained in the hindgut, or large intestine, which includes the cecum and colon. Microbes can be classified based on their fermentative activity or products of fermentation. Fibrolytic bacteria, such as *Fibrobacter* spp. or Ruminococcaceae members, ferment fibrous material, which is made up primarily of cellulose and hemicellulose. These species predominantly ferment fiber to produce volatile fatty acids (VFA), such as acetate, propionate and butyrate (Weimer, 1998; Daly et al., 2011). Acetate serves an energy source for many different tissues including the heart, skeletal muscle and brain. Propionate is the only gluconeogenic VFA and can be converted to glucose by the liver (Daly et al., 2001). Rather than being absorbed directly into the blood stream to be used as energy,

butyrate is metabolized primarily by the epithelium of the rumen and large intestine (Bergman, 1989). It plays an important role in epithelial health, differentiation and regulation (Daly et al., 2001). Saccharolytic species such as *Streptococcus bovis*, *Lactobacillus* spp., or *Bifidobacterium* spp. primarily ferment hydrolyzable carbohydrates such as amylose and amylopectin to produce predominately propionate and lactate (Nocek, 1997). Lactate-utilizing bacteria like *Megasphaera elsdenii* or *Selemonas ruminatium* ferment the lactate produced by saccharolytic microorganisms to form VFA. Propionate is the most common VFA produced by lactic acid fermentation, with some bacterial species being able to produce butyrate under certain conditions (Belengueret al., 2007; Duncan et al., 2004).

During the developmental period, the microbial populations of the gut are extremely sensitive to changes in the environment, which affects the well-being and performance of the host (Mackie et al., 1999; Chaucheyras-Durand and Durand, 2009). Stressors, such as the abrupt dietary change during weaning, will often lead to GI upset and diarrhea. This is likely due to decreased resistance to pathogens, due to compromised barrier function of the intestinal epithelium, and a population decline of *Lactobacillus* species, which are thought to facilitate the exclusion of pathogens (Abu-Tarboush et al., 1996; Krehbiel et al., 2003; Chaucheyras-Durand and Durand, 2009). Other stressors such as lactation, transportation, illness and antibiotic treatment can also alter the microbiome of the gut, leading to poor animal performance and increased morbidity and mortality (Krehbiel et al., 2003). Mitigation of some of the effects of these stressors will improve the productivity of the animal, in addition to promoting its overall health and welfare.

1.3 History of probiotic use

Supplementation of livestock with probiotics is aimed at promoting microbial balance and activity (Chaucheyras-Durand and Durand, 2009; Yirga, 2015). The first recorded use of live microorganisms as a feed supplement in livestock was the use of baker's yeast in pre-ruminant calves to supply vitamin B (Eckles et al., 1924). Supplementation of vitamin B through baker's yeast was expected to increase growth in calves. While treated calves did not grow more quickly, they showed improved health over their untreated counterparts with decreased diarrhea and improved resistance to disease (Eckles et al., 1924). Live yeast such as *Saccharomyces cerevisiae* (SC) continues to be the most commonly used probiotic in livestock production (Chaucheyras-Durand and Durand, 2009) .

Nurmi and Rantala (1973), examined the effect of treatment of immature broiler chickens with microflora isolated from the digestive tract of mature broilers, horse feces or bovine ruminal fluid. Chickens were then subjected to *Salmonella infantis* challenge. Supplementation with bovine or equine microflora had no effect on the ability of *S. infantis* to proliferate in the ceca of poultry. However, treatment with microflora cultured from mature broiler chickens prevented growth and colonization of *S. infantis* in the ceca, suggesting microbial supplementation may facilitate the exclusion of pathogens and reduce the transmission of disease in poultry.

"Probiotic" has been used in the United States as an umbrella term for supplements containing viable microbial cells, cellular products, enzymes, or growth media containing cellular metabolic products (Yoon and Stern, 1995). Due to the confusion caused by multiple meanings attributed to this term, the U.S. Food and Drug Administration (FDA) now requires the use of the term "direct fed microbials" (DFM) by manufacturers rather than "probiotic" to

identify products that contain naturally occurring, viable microorganisms (Yoon and Stern, 1995).

1.4 Mode of action

Direct-fed microbials are used to improve the health and performance of livestock through several modes of action. Live microbial cultures can be used to stimulate the immune system, reduce pathogen shedding, alter endogenous microbial populations and products, and increase digestibility of feedstuffs (Fuller, 1989; Fuller, 1999; O'Toole and Cooney, 2008; McAllister et al., 2011). Stimulation of the immune system and reduction of potentially zoonotic pathogens are clearly of great importance in food safety. This will become increasingly important following the implementation of the VFD as prophylactic use of antibiotics is further limited. The EU saw a significant increase in the incidence of foodborne disease following its ban on antibiotic use for growth promotion in 2006 (Collins et al., 2009; Gaggìa et al., 2010). The methods by which DFMs reduce pathogen shedding are under investigation and are dependent upon the specific microbe and strain being used.

Competitive exclusion is one method of pathogen inhibition by DFM (Fig 1.1). Supplying beneficial bacteria, such as *Lactobacillus* spp., to the animal creates competition with the pathogen. In the case of *Lactobacillus*, it is postulated that competition for nutrients and adhesion sites on the intestinal mucosa decreases the ability of pathogens, such as *Escherichia coli*, to establish in the gut (Blomberg et al., 1993; La Ragione et al., 2004). The concept of "colonization resistance" was introduced by van der Waaij (1971) to describe that a stable microbial environment in the GIT will prevent pathogenic invasion. Conversely, any disruption in microbial balance may increase an animal's susceptibility to pathogenic colonization.

Probiotics may also play an important role in immunomodulation. Because the GI

microbiome is the largest source of microbial stimulation of the immune system, establishment of a normal microbiological ecosystem shortly after birth is essential to the development of acquired immunity (Delcenserie et al., 2008). Dietary addition of probiotics increases natural killer cells and phagocytic activity of leukocytes in both humans and animals (Fuller, 1989; Gill, 2001). Direct fed microbial supplementation has also been reported to increase IgA production. This in turn, decreases the incidence of pathogenic colonization in the GIT. Lessard et al. (2009) supported this theory when they observed that piglets consuming a DFM of *Pediococcus acidilactici* and SC showed improved resistance to *E. coli* infection.

Production of antagonistic compounds such as bacteriocins and organic acids may be another mechanism by which DFM exclude pathogens. Bacteriocins are compounds secreted by bacteria that inhibit the activity of closely related bacteria. For example, enterocin, a bacteriocin produced by *Enterococcus faecium*, inhibits growth of listeria, staphylococci, enterococci and *E. coli* in the rumen (Laukova et. al., 1998).

Microbial diversity and balance also promotes the integrity of the intestinal epithelium to prevent the passage of pathogens and reduce inflammation (Salmimen et al., 1996; Gaggìa et al., 2010). Madsen et al. (2001) demonstrated that administration of a DFM containing *Bifidobacteria* and *Lactobacillus* spp. strengthened epithelial integrity and barrier function in mice with experimentally induced colitis. Treated mice displayed increased resistance to pathogenic infection and restoration of normal intestinal histology following disease challenge. Frank et al. (2007) reported that a disruption in microbial balance, such as a decrease in commensal bacterial populations, is linked with inflammatory bowel disorders in the human large intestine. Thus, restoration of microbial balance may serve to reverse or alleviate some intestinal epithelial damage due to chronic inflammation.

1.5 Use of direct fed microbials in swine production

Supplementation with DFM in swine production is most effective when animals are under physiological stress, such as during gestation, lactation, or weaning. Use of DFM in sows is often intended to mitigate weight loss during lactation and improve health status during both gestation and lactation. This reduces the sow's recovery time after farrowing, improves subsequent reproductive performance and results in improved piglet health. Supplementation with LAB, such as *E. faecium* or *Bacillus* spp., to sows during gestation and lactation increases feed intake and reduces weight loss associated with lactation (Alexopoulos et al., 2004; Böhmer et al., 2006). This is helpful in preventing "starvation sterility" which is the lengthened interval of time between weaning one litter and delivering the next caused by reduced feed intake and fat loss during lactation. "Starvation sterility" is a common problem in the swine industry, particularly affecting young sows after the birth of their first litter (Böhmer et al., 2006). Conversely, researchers studying effects of supplementation with DFM containing SC to sows found no differences in feed intake during lactation nor the length of the weaning to estrus interval compared to control sows. Sow body weights after lactation were also similar between treated and untreated sows (Veum et al., 1995; Jurgens et al., 1997).

Böhmer et al. (2006) found that sows administered a probiotic containing *E. faecium* had more piglets born alive and lost less weight during lactation than control sows. Supplementation with *E. faecium* also improved the sows' overall health in the peripartal period, thus decreasing the use of medication to treat illness. Use of this DFM may also improve piglet health and productivity. Alexopoulos et al. (2004) and Böhmer et al. (2006) reported that litter mass of sows supplemented with *E. faecium* tended to be greater at birth and weaning than that of their un-

supplemented counterparts. Supplementation of sows with DFM containing SC, however, did not affect litter size, piglet birth weight or weaning weight (Veum et al., 1995; Jurgens et al., 1997).

Piglets often experience diminished growth at weaning, attributed to physiological stress caused by the abrupt change in diet and environment. This stress results in anorexia, diarrhea and undernutrition. These animals are predisposed to GI disturbance and pathogenic colonization of the intestines (Bontempo et al., 2006). *Saccharomyces cerevisiae* administered to piglets during the peri-weaning period improves feed intake and average daily gain during this critical stage of development (Mathew et al., 1998; Bontempo et al., 2006). However, when Giang et al. (2010b) administered a DFM containing several strains of LAB to weaned pigs, no differences in growth performance were detected compared to controls.

Supplementation with probiotics may also promote the health of the GIT, helping to restore the integrity of the mucosa after the thinning that often occurs at weaning. Restoration of a healthy intestinal mucosa increases resistance to infection, thereby decreasing morbidity and mortality associated with weaning stress (Bontempo et al., 2006). Researchers found that supplementation of SC led to increased villus height and crypt depth in the intestinal mucosa while supplementation of *Lactobacillus brevis* increased the maturity of mucus secreting goblet cells in the duodenum and jejunum, indicating improved epithelial barrier function and health of GI tissue as a result of supplementation with the DFM (Bontempo et al., 2006; Davis et al., 2007). Administration of *L. brevis* also has been shown to decrease lymphocyte infiltration of the GIT; infiltration of lymphocytes causes inflammation and decreases animal performance. Goblet cell maturation, increased crypt depth and villus length and decreased inflammation of the GIT all contribute to a decrease in susceptibility to disease, an increase in absorptive capacity of the intestine, and increases in appetite, weight gain and body weight after weaning (Bontempo et al.,

2006; Davis et al., 2007). These findings are contradicted by Reiter et al. (2006) who reported no differences in villus height, crypt depth nor goblet cell numbers in GIT of pigs supplemented with a probiotic culture containing LAB compared to controls.

Direct fed microbials are also used to decrease diarrhea associated with opportunistic pathogens such as S*almonella* or *E. coli* thus decreasing morbidity and mortality. Researchers in several studies using LAB such as *Lactobacillus* spp., *Bacillus* spp., *Pediococcus* spp. and *E. faecium* reported improved fecal scores at weaning and increased feed efficiency in treated piglets compared to controls (Casey et al., 2007; Taras et al. 2007). Use of these DFM may also reduce the amount of *Salmonella* or enterobacteria shed in the feces, thus reducing disease transmission. Ross et al. (2009) found fewer Gram-negative pathogens present in the feces of pigs supplemented with a probiotic containing *Lactobacillus amylovorus* and *E. faecium.* These findings were supported by Casey et al. (2007) who reported fewer incidences of diarrhea and decreased *Salmonella* in the feces of pigs treated with a probiotic containing *Lactobacillus* and *Pediococcus* spp. Giang et al. (2010a) however, reported no differences in *E. coli* counts in the GIT of pigs that received a DFM containing a combination of LAB, including *L. acidophilus, P. pentosaceus* and *E. faecium.*

1.6 Use of direct fed microbials in poultry production

Benefits of probiotic supplementation in poultry include improved performance and health. Researchers have reported improvements in average daily gain, feed efficiency and exclusion of intestinal pathogens in response to supplementation with DFM. This may be accomplished through the activation of the immune system or by competitive exclusion. Salmonella is a common food-borne pathogen which populates the lower GIT of poultry and may be spread between birds through fecal transmission. Nayebpor et al. (2007) reported that

supplementation with Primalac, a DFM containing *Lactobacillus acidophilus, L. casei, Bifidobacterium thermophilum* and *E. faecium,* limited the growth of pathogenic bacteria like *Salmonella enterica* in broiler chickens by stimulating the immune system. Because activation of the immune system stimulates the production of antibodies, birds dosed with Primalac demonstrated increased macrophage and neutrophil populations to enhance natural killer T-cell activity (Nayebpor et al., 2007).

Supporting these data, Higgins, et al. (2007a) administered a DFM containing LAB to day-old broilers chicks within an hour of a challenge with *S. enterica* and *S. typhimurium* and found that 24 h following treatment the recovery of Salmonella from the ceca of treated birds was reduced when compared to controls. Higgins et al. (2007b) further explored this idea, supplementing LAB to day old broiler chicks within an hour of challenge with *S. enterica.* This stimulated an immune response, resulting in increased ileal macrophages and decreased *Salmonella* populations in the ceca within 24 h. The authors speculated that other factors, such as increased expression of interleukins, tumor necrosis factor alpha or immunoglobulins, influenced the decrease in pathogen populations observed in the broiler GIT.

Probiotics have been used to reduce fecal shedding of infectious agents and foodborne pathogens, which can result in the spread of disease among birds or to humans. *Bacilllus subtilis* is one such DFM used to decrease fecal shedding of foodborne pathogens. In a study by La Ragione and Woodward (2003), *B. subtilis* was administered to broiler chickens prior to a challenge with *Clostridium perfringens* and *S. enterica*. Birds dosed with the DFM shed less pathogens in the feces and exhibited reduced colonization of the pathogenic bacteria in the ceca when compared to their un-dosed counterparts. It was postulated that supplementation with *B. subtilis* interferes with the colonization of *S. enterica* and *C. perfringens.* Reduction in fecal

shedding of pathogens is important to maintaining flock health and preventing the spread of infection through the flock or into the food system.

In addition to improved GI health, feed efficiency can be positively affected by Primalac supplementation. This may be due to increased nutrient retention (Angel et al., 2005). Inhibition of pathogenic bacteria is thought to lead to increased energy availability and carbohydrate digestion by the normal bacterial communities of the large intestine, leading, in turn, to decreased feed to gain ratio (Nayebpor et al., 2007). Addition of the LAB contained in Primalac has also been shown to increase Ca, P and N retention in broilers (Angel et al., 2005). This reduces nutrient excretion, allowing for the use of diets with decreased nutritional profiles to meet the birds' metabolic needs. Feeding diets containing reduced nutrient levels may reduce the cost of feed and potentially decrease the environmental impact of broiler production (Angel et al., 2005). However, researchers have shown in several other studies that efficacy of supplementation with probiotics containing LAB in altering broiler performance is variable. Ahmad (2004) and Ergün et al. (2000) reported no differences in feed efficiency in birds that received the probiotic compared to controls. Ergün et al. (2000), also detected no differences in growth performance between treated and untreated broiler chickens.

1.7 Acidosis in ruminants and horses

In addition to providing health benefits by decreasing pathogenic infection, DFM have been used to improve performance in ruminants by altering microbial populations and products of fermentation in the GIT. Modification of ruminal fermentation may be advantageous in conditions that could induce acidosis in order to preserve health and productivity of the animal. With normal feed intake, ruminal production of organic acids will not exceed the rumen's absorptive capacity. Under these circumstances, microbial fermentation is considered stable and

the pH of the rumen undergoes minimal fluctuation. Average pH in the rumen is between 5.8 and 6.2, but can range from 5.6 to 6.5 (Nagaraja and Lechtenberg, 2007). Postprandially, pH may fall below 5.6 for a short time due to increased fermentative activity, but readily returns to the normal range (Nagaraja and Lechtenberg, 2007). Animals fed high-grain, low-roughage diets for a prolonged period of time, or those that are abruptly introduced to a high-grain diet, are at the greatest risk for acidosis (Nagaraja and Lechtenbereg, 2007; Gonzales et al., 2012). These feeding patterns can cause an accumulation of VFA or organic acids in the rumen, exceeding its buffering capacity. In turn, a reduction in pH is induced, decreasing the ruminal pH to suboptimal levels for many commensal microbes (Nagaraja and Titgemeyer, 2007). Acidosis can be divided into 2 categories, subacute ruminal acidosis (SARA) or lactic acidosis. Cattle fed high-grain diets for prolonged periods of time are at increased risk for lactic acidosis, also referred to as acute ruminal acidosis. Acute acidosis is marked by accumulation of lactic acid in the rumen, which causes ruminal pH to fall below 5.0. In contrast, SARA is defined as accumulation of VFA in the rumen which induces a prolonged period of suboptimal pH (5.0 to 5.5), decreasing the welfare and performance of the animal. Lactic acidosis is associated with diarrhea, dehydration, anorexia, damage to the ruminal mucosa, laminitis, liver abscesses, long term depression of performance and in some cases, death (Nocek et al., 1997; Owens et al., 1998; Nagaraja and Lechtenberg, 2007; Nagaraja and Titgemeyer, 2007; Gonzalez et al., 2012). The signs and symptoms of SARA are less apparent than those of lactic acidosis and may be difficult to recognize immediately following onset. Symptoms of SARA become more evident over time. In dairy cattle, clinical symptoms of SARA are decreased milk production, decreased feed intake and signs of gastrointestinal discomfort, while in beef cattle, reduced feed intake and decreased performance are primary indicators of SARA (Nagaraja and Titgemeyer, 2007). In

order to minimize the decline in pH as well as the microbial imbalance corresponding with increased dietary inclusion of grain, DFM may be provided. One example of this application is the supplementation of SC to dairy cows during a subacute ruminal acidosis (SARA) challenge. Cows supplemented with SC experienced a less dramatic decline in ruminal pH and spent less time with a ruminal pH below 5.6 than controls (Chiquette, 2009).

Another common method to minimize the risk of ruminal acidosis is increasing the length of time spent acclimating the animal to a high-concentrate, low-forage diet. A normal adaptation period to a high-grain diet (90% concentrate) in feedlot cattle for the maintenance of ruminal health is between 21 and 28 d (Bevans et al., 2005). In horses, to maintain cecal health, the commonly recommended adaptation period to a high-concentrate diet is between 10 and 21 d with the amount of concentrate provided to the animal increasing by small increments each day. Adaptation to high-grain diets is meant to allow colonies of lactate-utilizing bacteria (LUB) to establish sufficient populations in order to metabolize the increased concentrations of lactate produced by amylolytic bacteria when the host is consuming a high-grain diet, thereby preventing lactate accumulation and subsequent acidosis (Nagaraja and Lechtenberg, 2007). In ruminants fed diets rich in starch without sufficient adaptation time, concentrations of LAB such as *S. bovis* experience rapid proliferation. The resulting explosive increase in fermentation causes a decline in ruminal pH, inhibiting the growth of many ruminal microorganisms. Growth of *S. bovis* begins to decline as pH falls below 6.0. *Lactobacillus* spp., which are more acid tolerant than *S. bovis*, become the predominant ruminal LAB as pH continues to decrease below 5.0. Once the pH in the rumen falls below 5.0, the animal is considered to be acutely acidotic (Nagaraja and Titgemeyer, 2007). At this pH, death and lysis of fibrolytic bacteria LUB occur. The decline of *S. bovis* in concurrence with the growth of *Lactobacillus* spp. cannot be solely

attributed to their differences in acid tolerance. In fact, Wells et al. (1997) determined that *L. fermentum* produces a bacteriocin that inhibits *S. bovis*. Although *S. bovis* is considered the major etiological agent of acidosis, once the pH falls below a certain threshold, *Lactobacillus* spp. are established as the major LAB in the rumen, as indicated in Fig 1.2 (Nagaraja and Lechtenberg, 2008).

The equine hindgut is similar in function to the bovine rumen, serving as the primary site of microbial fermentation in the GIT. Although the primary goal of altering ruminal fermentation in cattle is to improve performance, in equine production improvement of growth performance and feed efficiency is of lesser priority. Equine owners and researchers are chiefly concerned with improving the welfare of their animals by increasing horse health and longevity. Although horses are adapted to continuously grazing fibrous forage, modern equine management often disrupts natural feeding patterns with the addition of starch-rich concentrates (Daly et al., 2012). Starch consumption by horses in excess of 0.2 to 0.4 % of BW per meal, depending upon the chemical composition of the starch and the processing of the grain, exceeds the enzymatic digestive capacity of the small intestine. If starch escapes digestion and absorption in the small intestine, it will pass into the hindgut where it will undergo rapid and unchecked microbial fermentation (Potter et al., 1992; Kienzle, 1994).

Although starch is at least partially digested in the small intestine, soluble fiber, resistant starches and oligosaccharides resist enzymatic digestion in the small intestine and are delivered intact to the large intestine for fermentation. Like starch, these carbohydrates are rapidly fermented in the cecum and colon. For this reason, Hoffman et al. (2001) suggested a new classification system of carbohydrates specific to the digestive system of the horse (Fig 1.3). This system divides carbohydrates into hydrolyzable, rapidly fermentable and slowly fermentable

fractions. Hydrolyzable carbohydrates (CHO-H) include mono and di-saccharides, some oligosaccharides and starch. Rapidly fermentable carbohydrates (CHO-F_R) include resistant starches, some oligosaccharides and soluble fiber. Slowly fermentable carbohydrates (CHO-F_S) include hemicellulose and cellulose. Fermentation of CHO-F_R leads to the same decrease in cecal pH and overproduction of VFA and lactate seen in bacterial fermentation of CHO-H (Hoffman et al., 2001). As the pH of the hindgut decreases, protozoal populations begin to decline as well (Goodson et al., 1988). Introduction of starch or soluble fiber to the hindgut favors the proliferation of Gram-positive, LAB species such as *S. bovis, S. equinus* or *Lactobacillus* spp. (Milinovich et al., 2007; Daly et al., 2012). As the proliferation of LAB occurs, populations of acid intolerant, fibrolytic bacteria such as *Ruminoccocus* spp. decrease (Daly et al., 2012). Lactate utilizing bacterial populations initially remain unchanged, before increasing about 7 d after the introduction of starch or soluble fiber (Goodson et al., 1988). Decreased pH and increased lactate production associated with proliferation of *Streptococcus* spp and *Lactobacillus* spp. has been linked to the onset of several secondary diseases and disorders (Garner et al., 1978; Shirazi-Beechey, 2008).

Clinical signs of acidosis in cattle are largely mirrored in equines when abruptly exposed to large amounts of CHO-H or CHO-F_R, such as high-concentrate diets or lush pasture. The process by which lactic acidosis occurs has been more extensively documented in the ruminant than in the horse. Due to the similarity in the microbial environments of the rumen and the cecum of the horse, parallels may be drawn between these species in the development of this disorder and subsequent secondary issues (Garner et al., 1977; Milinovich et al., 2010).

1.8 Secondary issues caused by lactic acidosis in horses

Several comorbidities are often observed with acidosis and may persist even after the pH of the rumen or hindgut returns to normal. One such condition is laminitis which is considered one of the most serious conditions of the equine foot, often necessitating euthanasia (Milinovich et al., 2008; Al Jassim and Andrews, 2009). Although the exact mechanism behind dietary induced laminitis is debatable, the underlying cause is over consumption of hydrolyzable and rapidly fermentable carbohydrates.

Garner et al. (1978) found that a reduction in Enterobacteriaceae populations occurred with the onset of laminitis in response to reduced hindgut pH, which was associated with proliferation of Streptococci and Lactobacilli. They postulated that depression of pH induced lysis of Enterobacteriaceae bacterial species, which resulted in the release of endotoxins into the cecum. Endotoxins enter the blood stream, a process facilitated by mucosal damage in the hindgut caused by lactate accumulation and the associated decrease in pH. Vascular endotoxins are thought to induce lamellar separation in the hoof (Garner et al., 1978). Alternatively, Milinovich et al. (2007) observed a significant increase in streptococci populations concurrent with the onset of laminitis and postulated that *Streptococcus* spp., rather than Enterobacteriaceae, are the causative agents of laminitis. Both lactobacilli and streptococci can decarboxylate amino acids to produce amines, possibly as an internal defense mechanism in response to low pH (Bailey et al, 2004). Bailey et al., (2002) reported that amines cause vasoconstriction of digital blood vessels *in vitro,* and are increased in carbohydrate overload conditions (Bailey et al., 2002). It was therefore hypothesized that amines act as a pharmacological mediator of cecal pH by preventing drastic pH decline, and may be responsible for lamellar degeneration.

Colic, the predominant cause of emergency treatment in horses and second only to old age in cause of death, is directly linked to carbohydrate overload of the hindgut (Shirazi-Beechey, 2008). Colic is Reduced cecal pH caused by carbohydrate overload, coupled with lactate accumulation, is commonly associated with decreased fluid content of the digesta, which results in an increased risk of colonic impaction. Lactate producing bacteria, which proliferate in response to carbohydrate overload, also produce large amounts of CO_2 which can lead to other forms of colic (Shirazi-Beechey, 2008).

Proliferation of LAB occurs at the expense of acid-intolerant fibrolytic species creating microbial imbalance (Daly et al., 2012). This causes a shift in microbial fermentation products, namely a decrease in the acetate: propionate ratio (A:P). Butyrate production is also decreased, which has a deleterious effect on the health of the cecal epithelium. Reduced butyrate and A:P are associated with decreased GI health, and indicative of impaired microbial balance where diversity of microorganisms and populations of fibrolytic bacteria decrease in favor of LAB (Shirazi-Beechey, 2008). The shift in microbial populations induced by feeding diets high in CHO-H and CHO-F_R results in reduced fermentation of fiber and increased lactate production in the hindgut (Potter et al., 1992; Medina et al., 2002; Harlow et al., 2016). Intervention methods may be used to mediate the negative effects caused by inclusion of dietary starch or CHO-F_R.

1.9 Current methods of treating and controlling acidosis

The simplest method of preventing acidosis in the equine is simply to decrease the CHO-F_R or CHO-H in the diet. However, this is not an option if the energy requirements of the horse cannot be met without the inclusion of these products. Botanical origin, chemical composition of starch, and processing of the feed are major factors in determining starch absorption in the small intestine (Julliand et al., 2006). Feeding grains with greater prececal starch digestibility, or

processing grain to improve prececal starch digestibility and absorption, is an alternative to decreasing the amount of grain fed, while decreasing the incidence of acidosis and providing sufficient energy. Mechanical processing such as grinding or rolling alters the physical form of the starch granules, increases the surface area, and thereby enhances the enzymatic digestion of starch in the small intestine. Thermal and hydrothermal processing increases the extent of starch gelatinization, thus increasing its solubility and susceptibility to prececal enzymatic digestion (Julliand et al., 2006). Oat starch has the greatest prececal digestibility regardless of processing, and is generally regarded as safer for consumption by the equine compared to corn or barley (Kienzle et al., 1992; Harlow et al., 2016).

Use of antibiotics to alter microbial fermentation has been proven effective in the prevention of cecal acidosis in horses. Virginiamycin is an antibiotic produced from *Streptomyces virginiae* that inhibits protein synthesis of Gram-positive bacteria (Nagaraja, 1995). Fecal pH of horses dosed with virginiamycin either prior to, or within 8 h following excess grain consumption, was markedly greater than that of untreated animals (Rowe et al., 1994). D-lactate concentrations were also greater in untreated horses compared to the control. Researchers concluded that virginiamycin is an effective means of preventing dramatic pH reduction in the hindgut through inhibition of LAB. Bailey et al. (2002) determined that virginiamycin decreases the production of vasoactive amines in response to starch or fructan *in vitro*. As a result, it may be used to reduce the incidence of laminitis (Rowe et al., 1994). In fact, a product containing virginiamycin called Founderguard ® is available for prevention and treatment of laminitis in Australia and New Zealand.

Other methods of altering microbial fermentation have also been used in the horse including supplementation with prebiotics and DFM. Prebiotics have been used to improve

health and welfare in livestock by promoting balanced microbial populations and fermentation (Respondek et al., 2008). Short and medium chain fructooligosaccharides (FOS) are the most commonly used prebiotics (Berg et al., 2005). These oligosaccharides escape enzymatic digestion of the small intestine and are fermented in the hindgut (Hoffman et al., 2001). Prebiotics improve gut health by promoting the growth of beneficial bacteria such as bifidobacteria and lactobacilli (Respondek et al., 2008). In a study conducted by Berg et al. (2005), feeding yearling horses high and low doses of FOS without the addition of dietary starch resulted in a linear decrease in fecal pH and an increase in VFA and lactate production as prebiotic dosage increased. A quadratic effect on *E. coli* populations was observed with the low dose of FOS causing a decrease in *E. coli* concentration. Lactobacilli concentrations also increased with addition of FOS to the diet, although researchers were unable to enumerate bifidobacteria. Researchers concluded that offering FOS as a prebiotic energy source for beneficial bacterial populations can increase VFA production, decease pathogen colonization and improve microbial balance in the hindgut of the horse (Berg et al., 2005).

Prebiotics have been evaluated for their potential to mitigate the risk of acidosis following excess starch intake as well. Respondek et al. (2008) reported that when fed at a level of 30 g/d, addition of dietary FOS appeared to reduce the negative effects of starch overload. *Lactobacillus* and *Streptococcus* populations remained stable even after starch levels increased, and lactate did not accumulate in the hindgut of horses supplemented with FOS. The pH of the hindgut remained stable in FOS supplemented horses compared to the decrease noted in control animals. Cecal concentrations of acetate and butyrate also tended to increase in FOS supplemented horses (Respondek et al., 2008). Increased butyrate production is associated with improved health of the intestinal epithelium, while the establishment of the pathogenic bacteria,

E. coli, has been associated with increased streptococci and lactobacilli production (Milinovich et al., 2007). Supplementation of horses with prebiotics such as FOS may be a useful tool in promoting gut health as well as decreasing risk of disease or disorders linked to grain feeding in horses.

1.10 Use of direct fed microbials in horses

In addition to improving the growth of beneficial microbes indirectly through prebiotic supplementation, beneficial microorganisms can be fed directly to the animal to promote gut health. *Saccharomyces cerevisiae* is the most common DFM used in horses to alter microbial fermentation. Yeast must be consumed daily to be effective, because, although it will survive for a short time in the equine GIT, it will not colonize (Schoster et al., 2014). In a study by Medina et al. (2002), SC was administered to horses receiving either a high fiber (HF) or high starch (HS) diet. While SC had no effect on total microbial counts, supplementation did alter the proportions of some bacterial populations. A positive effect of SC supplementation on lactobacilli concentrations and a negative effect on streptococci concentrations was observed in horses fed the HF diet. These results were supported by Jouany et al. (2009) who observed a trend for increased lactobacilli and LUB in horses supplemented with SC. In horses fed HS diets, SC supplementation appeared to prevent the dramatic reduction in cecal pH often associated with increasing levels of starch in the diet (Medina et al., 2002). This may be a result of reduced lactate concentration in the hindgut of SC supplemented horses.

Yeast has also been used to alter the digestibility of substrates in the hindgut. Improved fiber digestion has been attributed to SC supplementation. In a study performed by Jouany et al. (2008) ADF digestibility increased with daily SC supplementation in horses fed either HF or HS diets. Dry matter and NDF intake were also stimulated by the inclusion of this DFM, although

the effect was greater for horses consuming HF than HS diets. It was determined that increased fermentation of fiber observed with SC supplementation was due to stimulation of microbial cellulolytic activity rather than an alteration in bacterial populations, as concentrations of cellulolytic bacteria in the cecum were not affected by supplementation of SC. In a follow-up study, Jouany et al. (2009) determined that the primary mode of action of SC to increase ADF digestibility is the promotion of fibrolytic enzymatic activity. Addition of SC to the diet resulted in improved microbial β-D-cellobiodase, β-D-glucosidase, xylanase, arabinosidase and β-D-xylosidase activity in the hindgut (Jouany et al., 2009). Supplementation of SC could be a viable strategy to improve nutritional status of horses by improving fiber digestion. However, SC supplementation is inconsistent in delivering these results. In other studies, dietary SC addition did not affect pH, microbial populations or fibrolytic activity in the equine hindgut (Taran et al., 2016). A combination DFM with *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum* and *Enterococcus faecium* increased lipid digestion by 5.1%, as well as increasing digestibility of copper and iron (Swyers et al., 2008). Other probiotics have been tested in horses with inconclusive results. In several studies where horses were administered a DFM containing SC, *Lactobaccilus* or *Enterococcus* spp. researchers reported no differences between controls and DFM supplemented horses in *Salmonella* shedding, diarrhea, or morbidity (Parraga et al., 1997; Kim et al., 2001; Boyle et al., 2013). Lattimer et al. (2007) found no differences in nutrient digestibility, fermentation or microbial populations when comparing *in vitro* models of equine fecal cultures with or without SC inclusion. Contrary to previous studies showing improved GI health in horses supplemented with DFM containing LAB, Weese et al. (2005) found that administration of a DFM containing *L. pentosus* to newborn foals was associated with increased colic, anorexia, diarrhea and need for veterinary intervention. It is evident that the effects of

DFM supplementation in horses are inconsistent and require further research (Schoster et al., 2014).

1.11 *Megasphaera elsdenii*

Megasphaera elsdenii is a commensal microbe in the GIT of monogastric animals. However, its use as a probiotic in monogastrics is relatively unexplored. This large, Gram-negative coccus is the primary LUB of the GIT, fermenting between 60 and 80% of the lactate produced in the rumen and presumably in the cecum (Counotte et al., 1981). Excess lactate production, typically induced by rapid transition to high-grain diets, decreases the pH of the microbial environment and puts the animal at risk of acidosis. *Megasphaera elsdenii* is viable at a pH below 5.5, which is inhibitory to other LUB such as S*elemonas ruminatium* and *Veillonella* spp. (Lewis and Elsden, 1955). In fact, Therion et al. (1982) reported that *M. elsdenii* is able to grow over a broad pH range, from 4.6 to 7.8, with an optimal pH of about 6.05. *Megasphaera elsdenii* does not experience catabolic repression in the presence of glucose as other lactate fermenters, such as *Selemonas ruminatium*, are known to do, affording *M. elsdenii* a competitive advantage over these microorganisms. In fact, *M. elsdenii* can ferment glucose to produce VFA (Hino and Kuroda, 1993; Weimer and Moen, 2013). In addition to the ability to withstand acidic pH and variation in substrates, *M. elsdenii* is more oxygen tolerant than other LUB (Meissner et al., 2010). This hardy bacterium is also ionophore resistant (Callaway et al., 1999), expanding its potential for use in ruminant livestock routinely supplemented with ionophores.

Although *M. elsdenii* can utilize glucose and lactate simultaneously as substrates for VFA synthesis, it will preferentially metabolize lactate when it is available in concentrations greater than 1 to 2 mM (Hino et al., 1994; Marounek et al., 1989). The ability of *M. elsdenii* to metabolize glucose allows it to compete for substrate with LAB such as *Streptococcus bovis* if

lactate is not available in sufficient supply. Hino et al. (1994) reported that growth of *M. elsdenii* is greatly influenced by the lactate production of *S. bovis.* Lactate is fermented by *M. elsdenii* into propionate at a rate 5 times faster than fermentation of glucose into VFA (Hino et al., 1994). This LUB produces propionate via the acrylate pathway (Fig 1.4), rather than the more common succinate pathway used by most other LUB to ferment lactate into propionate (Counette et al., 1981; Prabhu et al., 2012). Fermentation of lactic acid into propionate provides energy to the host and aids in stabilization of the environmental pH to prevent acidosis.

Megasphaera elsdenii also is important in the maintenance of mucosal health. Butyrate production from lactate or glucose by this organism under acidic conditions facilitates redox balance, consuming NADH without utilizing ATP (Figure 1.4; Counette et al., 1981; Slyter et al., 1992; Prabhu et al., 2012). In rats, *M. elsdenii* stimulates butyrate synthesis while balancing excess lactate production in the large intestine (Hashizume et al., 2003). Lactate production was activated in the large intestine by feeding a FOS supplemented diet *ad libitum* which, in turn, induced diarrhea in all rats. However, those dosed with *M. elsdenii* 4 d after FOS introduction recovered more quickly than control animals and tended to have less fecal moisture during diarrheal episodes. They also exhibited greater concentrations of butyrate in the feces compared to controls which is associated with improved health of the GIT. Large intestinal mucosa samples revealed greater epithelial cell numbers and thicker mucosa in supplemented animals over controls (Hashizume et al., 2003).

Proteolytic activity of *M. elsdenii* has also been documented. Early work by Lewis and Elsden (1955) demonstrated the fermentation of L-Serine, L-Threonine and L-Cysteine by *M. elsdenii.* Production of isobutyrate, isovalerate, and valerate is also indicative of proteolytic activity (Allison, 1978). *In vitro* studies of lactate fermentation revealed increased production of

isobutyrate, butyrate, valerate and isovalerate, as well as decreased lactate accumulation, in response to inoculation with *M. elsdenii* (Kung and Hession, 1995). Deamination of branched-chain amino acids to produce branched-chain fatty acids by proteolytic bacteria such as *M. elsdenii* provides an important growth factor for cellulolytic bacterial (Miura et al., 1980). Additionally, caproic acid production by *Megasphaera* has been observed as a result of protein or glucose fermentation (Marounek et al., 1989; Shetty et al., 2013). Caproate is thought to suppress pathogen colonization in the large intestine (Immerseel et al., 2004). *In vitro* synthesis of B vitamins by *M. elsdenii* has also been reported (Shetty et al., 2013).

1.12 Use of *Megasphaera elsdenii* in ruminants

In recent years, *M. elsdenii* has been evaluated for its potential use in ruminant livestock. The ability of *M. elsdenii* to prevent lactate accumulation and excessive decline in pH in *in vitro* models of acute acidosis has been reported. Acidosis can be simulated *in vitro* by the addition of a slurry containing high levels of hydrolyzable carbohydrates to ruminal fluid obtained from a steer previously adapted to a 100% forage diet (Kung and Hession, 1995; Long et al., 2014). Kung and Hession (1995) found that, at a dosage of 8.7×10^6 CFU/mL of culture fluid, *M. elsdenii* was successful in preventing lactic acid accumulation. In fact, the concentration of lactic acid never increased above 2 mM following dosing and acidosis challenge. This is well below the 50-mM threshold established by Nagaraja and Titgemeyer (2007) as indicative of the onset of acute acidosis. *Megasphaera elsdenii* also prevented extreme pH reduction following the introduction of hydrolyzable carbohydrates. While the pH of untreated cultures decreased to a minimum of 4.8 (Nagaraja and Titgemeyer, 2007), the pH of cultures treated with *M. elsdenii* never fell below 5. These findings were supported by Long et al. (2014) who observed an acid

tolerant strain of *M. elsdenii* prevented excessive pH decline and lactate accumulation *in vitro* following acidosis challenge.

In order to determine the viability and effect of *M. elsdenii in vivo,* Klieve et al. (2003) inoculated steers with a culture containing *M. elsdenii* via a rumen cannula prior to an abrupt transition from a forage-based to concentrate-based diet. While no differences in ruminal pH, VFA concentrations or VFA profile were detected between control and inoculated cattle, *M. elsdenii* immediately established ruminal populations after inoculation. Wild type *M. elsdenii* eventually populated the rumens of untreated animals, however, a similar population density to that of the inoculated animals was not established until about 12 d after the introduction of grain (Klieve et al., 2003). The authors hypothesized that the early establishment of this LUB may be beneficial in preventing lactate accumulation in animals predisposed to lactic acidosis. This idea was further investigated with the intra-ruminal administration of *M. elsdenii* to sheep and cattle in several studies by Henning et al. (2009; 2010) to determine the effect on productivity and ruminal characteristics under carbohydrate challenge. Neither sheep nor cattle that received *M. elsdenii* experienced as dramatic of a reduction in ruminal pH as the untreated animals. Inoculation with *M. elsdenii* decreased the amount of time ruminal pH of the rumen remained under 5.5 and led to decreased lactate accumulation. Additionally, *M. elsdenii* has been shown to promote feed intake, with both sheep and cattle consuming more concentrate and less forage than their untreated counterparts, with no detrimental effect on productivity. In fact, average daily gain of cattle was improved with the addition of the probiotic culture (Henning et al., 2010). Since roughage is an expensive energy source, the ability to utilize less roughage and hasten the transition to finishing diets without negatively affecting productivity would be economically beneficial to producers.

Oral supplementation of *M. elsdenii* to feedlot cattle has been shown to ameliorate the detrimental effects of an abrupt transition to a high concentrate diet. Cattle that received *M. elsdenii* tended to better cope with abrupt introduction of a finishing feedlot ration. Heifers rapidly stepped up to finishing diets performed equivalently to control animals managed under a more traditional, gradual transition from forage to concentrate based diets (Miller et al., 2013). In another study by Miller et al. (2013), administration of *M. elsdenii,* along with an abrupt transition to a high concentrate finishing diet, resulted in decreased dry matter intake compared to the control during the first 18 d after dietary transition, contradicting Henning et al (2010). However, there were no differences in ADG or carcass characteristics in rapidly transitioned animals that received *M. elsdenii* when compared to animals which received no probiotic and were stepped up gradually to the finishing diet. Fecal output also decreased and the overall digestibility of feed tended to be greater in animals that received the DFM (Miller et al., 2013). This increase in digestibility of feedstuffs and the associated decrease in fecal output may help to reduce the environmental impact of cattle production. The abundance of liver abscesses or diagnosis of acidosis also did not vary between treatments (McDaniel et al., 2009; Miller et al., 2013). Diarrhea and bloat are often most problematic in feedlot cattle immediately following transition to high concentrate diets. Cattle treated with *M. elsdenii* tended to experience decreased occurrences of both diarrhea and bloat, and overall decreased morbidity (Leeuw et al., 2009). These animals exhibited increased average daily gain over than their untreated counterparts immediately after the adaptation period, and experienced fewer health issues than control cattle. The ability to decrease the amount of roughage necessary in ruminant diets and minimize the length of the transition period necessary to adapt cattle to a finishing diet will have significant economic implications for producers. Mitigation of adverse effects of high

concentrate diets and a reduction in overall morbidity can presumably increase animal performance while decreasing the cost of maintenance of animal health and welfare.

In dairy cattle, supplementation with *M. elsdenii* resulted in decreased fecal starch concentration, possibly due to increased ruminal fermentation (Hagg et al., 2010). *Megasphaera elsdenii* has not been shown to affect milk yield, body weight, or amount of time spent eating or ruminating in dairy cows (Hagg et al., 2010; Aikman et al., 2011). However, the ability of this LUB to stabilize ruminal pH and reduce the amount of time the rumen spends under SARA conditions (pH < 5.6) may improve production efficiency of these cows (Aikman et al., 2011). It was also hypothesized that the increase in propionate production, seen in the rumen of cows that received *M. elsdenii,* may positively impact the energy balance of the cow due to its gluconeogenic nature, which could potentially improve milk production efficiency (Aikman et al., 2011). Aikman et al. (2011) reported production efficiency increased by 0.3 kg milk/kg of DMI in dosed cows compared to those that did not receive *M. elsdenii.* However, Aikman also reported depressed milk fat concentrations in cows that received *M. elsdenii* compared to control cows. Health of dairy cows in this study was positively affected by supplementation with *M. elsdenii* which supports data reported by Leeuw et al. (2013) in beef cattle. Hagg et al. (2010) reported that morbidity in dairy cattle decreased by approximately 50%, with fewer cows needing to be culled in supplemented groups compared to controls.

Neonatal ruminants have a predominantly sterile gut environment at birth, as well as an undeveloped rumen; this is referred to as the pre-ruminant stage. In calves, the purpose of DFM supplementation is to establish beneficial intestinal microflora and promote development of the rumen, rather than improving existing ruminal function (Fuller, 1999; McAllister et al., 2011). *M. elsdenii* supplementation of neonatal dairy calves evoked the desired effect, hastening

reticulorumen development. Calves that received the bacterium displayed greater reticulorumen weight, as well as greater papillae width and density on the ruminal mucosa than control calves (Muya et al., 2015). Butyrate production also increased in supplemented animals, yielding greater β-hydroxybutyrate in the blood stream. β-hydroxybutyrate is a product of the epithelial metabolism of butyrate. The presence of this product indicates increased ruminal epithelial metabolism, leading to an increased rate of development of rumen function, as well as improved absorption of nutrients in calves supplemented with *M. elsdenii* (Muya et al., 2015).

1.13 Use of *Megasphaera elsdenii* in monogastrics

Use of *M. elsdenii* as a DFM in monogastric animals is relatively unexplored. Hashizume et al. (2003) assessed the ability of *M. elsdenii* to alleviate the effects of hyper-lactate production stimulated by FOS in rats, and established that the bacterium can decrease fecal lactate concentration. Rats supplemented with *M. elsdenii* also recovered more quickly from hyper-lactate induced diarrhea. Administration of *M. elsdenii* resulted in an increase in butyrate concentration in the cecum which, in turn, stimulated an increase in cecal epithelial cell numbers (Hashizume et al., 2003).

Researchers found in an *in vitro* model of the porcine cecum with added sodium gluconate, butyrate and valerate concentrations increased when *M. elsdenii* was added, especially when cultured with *Lactobacillus acidophilus* (Tsukahara et al., 2006), leading to the hypothesis that administration of these microorganisms may improve piglet mucosal recovery after weaning. Intestinal villus atrophy, and decreased immunoglobulin A (IgA) production are commonly observed in piglets post-weaning. When a DFM containing *L. acidophilus* and *M. elsdenii* was administered to piglets weaned at 20 d of age, colonic butyrate concentration increased, corresponding with greater colonic mucosal thickness (Yoshida et al., 2009). In addition to

increased mucosal thickness, villous height was greater in DFM treated piglets. This indicates accelerated recovery from villous atrophy in piglets that received the probiotic. Expeditious mucosal recovery from weaning is associated with enhanced nutrient absorption as well as improved epithelial barrier function (Yoshida et al., 2009). Intestinal IgA concentrations also tended to increase in piglets that received *L. acidophilus* and *M. elsdenii*, signifying improved immune function over control piglets. These piglets also recovered more rapidly from diarrhea associated with weaning (Yoshida et al., 2009).

In an equine *in vitro* study, which simulated cecal acidosis using either corn starch or fructans to induce acidotic conditions, researchers noted that the addition of *M. elsdenii* to these cultures decreased lactate accumulation and minimized pH decline (Leventhal et al., 2011). Researchers also reported increased acetate, valerate and butyrate production in response to *M. elsdenii*, as well as improved fermentative activity. Leventhal et al. (2011) postulated that *M. elsdenii* may have the potential for use in the equine to minimize lactate accumulation under conditions that could lead to acidosis, such as over-consumption of grain or grazing on lush spring pasture. Researchers established the survivability of this bacterium through the equine stomach and small intestine to reach the hindgut when the probiotic culture was administered orally in an unpublished trial conducted at Kansas State University (Dove et al., unpublished) The effect of the administration of *M. elsdenii* on the equine hindgut has not yet been evaluated *in vivo*.

In two unpublished studies conducted at Kansas State University, researchers found that *M. elsdenii* provision to broiler chickens either as an oral gavage or lyophilized feed additive, increased feed efficiency up to 4.6% during the first 3 wk of life. A follow-up commercial study reported that broiler feed conversion rates improved as much as 5.5% in response to *M. elsdenii*

supplementation either as an aerosolized mist or oral gavage (Drouillard, personal communication).

Further studies examining the effect of supplementation with *M. elsdenii* on these monogastric species have not yet been published.

1.14 Summary

As FDA regulations become more stringent regarding antibiotic inclusion in livestock diets, it is imperative to identify new methods to improve livestock health and performance. Supplementation of livestock and poultry with DFM is a promising alternative. Some have shown probiotics to be an effective means of improving the health status and productivity of domestic livestock and poultry. Direct administration of beneficial microorganisms has also been successful in alteration of microbial fermentation and improvement of gastrointestinal health in horses. A DFM containing lactate-utilizing *M. elsdenii* has potential for use in modern production systems. This DFM may provide alternatives to antibiotics in improving performance and health status in swine and poultry and in reducing issues associated with feeding high-concentrate diets in equines. Thus, the following studies were conducted to determine the effect of *M. elsdenii* supplementation on poultry performance and cecal parameters, sow and piglet performance and fecal parameters, and equine cecal parameters when provided a high concentrate diet.

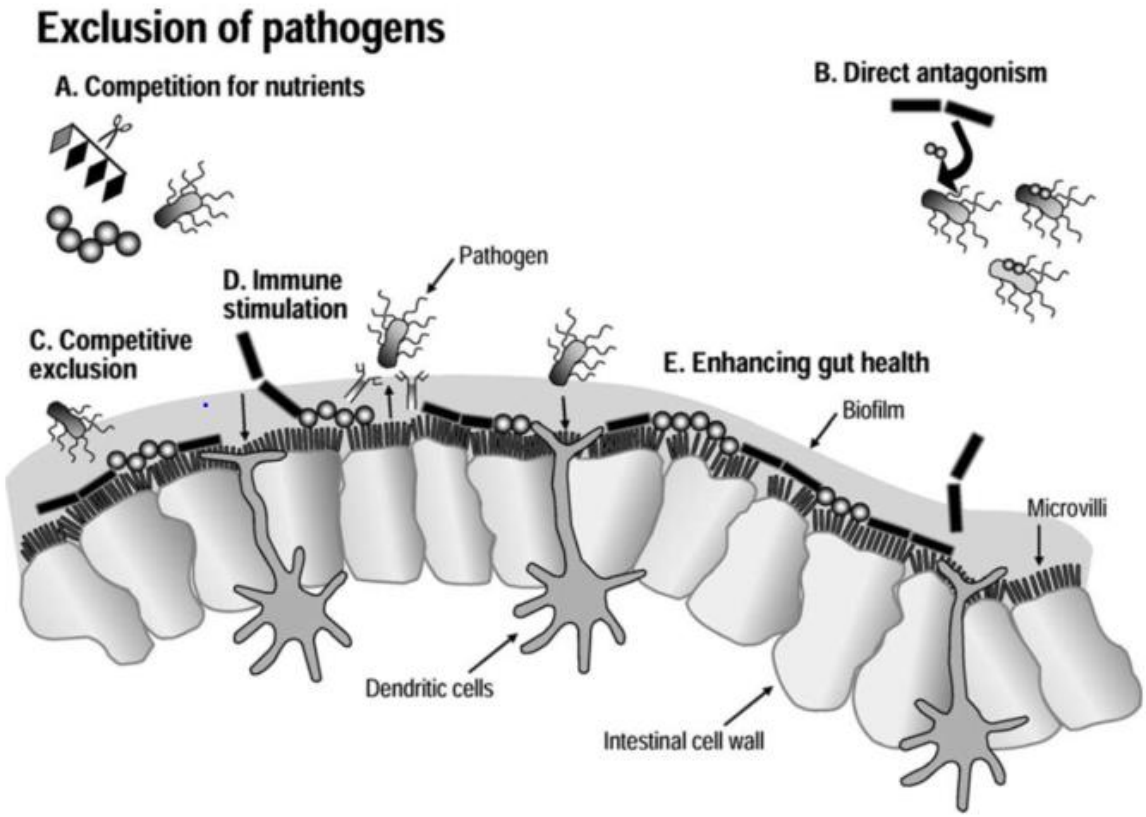

Figure 1.1 Demonstration of mechanisms by which direct fed microbials (DFM) competitively exclude pathogens from the gastrointestinal tract. (A) Limitation of microbial growth through competition for nutrients (e.g., Zn +2); (B) Production of antimicrobials such as bacteriocins, antibiotics or organic acids; (C) Prevention of adhesion through competition for binding sites; (D) Stimulation of host immune response for pathogenic exclusion; (E) Restoration and maintenance of epithelial integrity (McAllister et al., 2011).

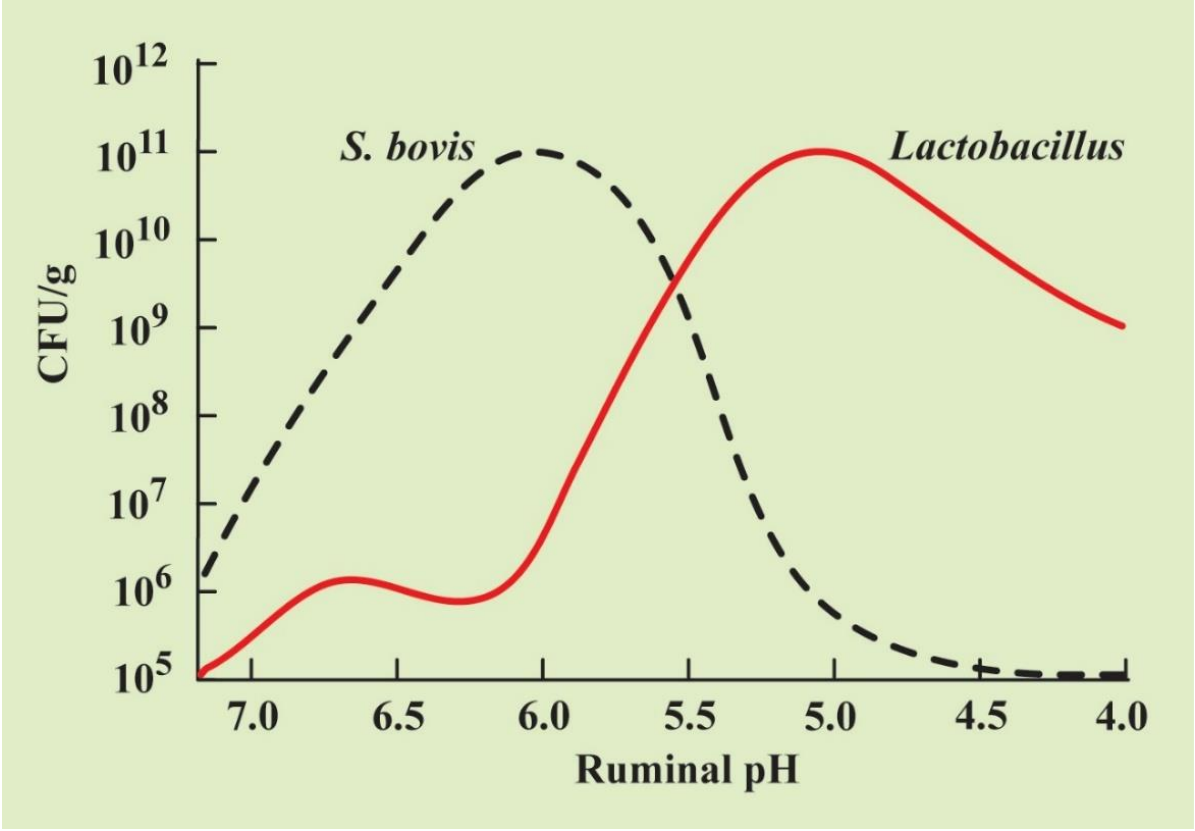

Figure 1.2 *Streptococcus bovis* and *Lactobacillus* spp. concentrations in response to changes in ruminal pH. (From Nagaraja, T.G.G., 2016; with permission)

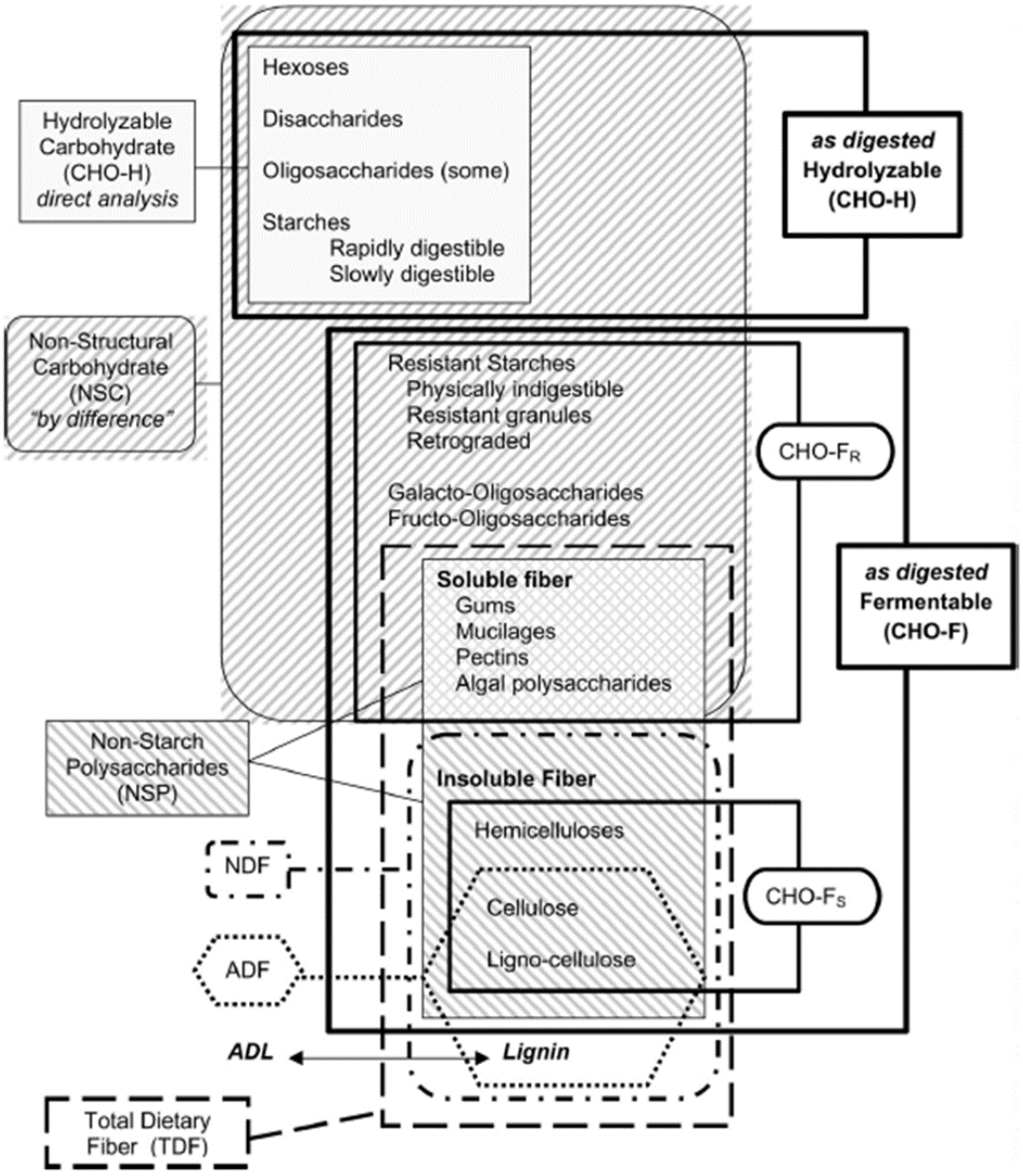

Figure 1.3 Carbohydrate partitioning specific to the equine gastrointestinal tract compared to traditional proximate analysis partitioning of carbohydrates (Hoffman et al., 2001). This system divides carbohydrates into hydrolyzable carbohydrate (CHO-H), rapidly fermentable carbohydrate (CHO-FR) and slowly fermentable carbohydrates (CHO-FS) fractions (right) compared to traditional proximate analysis fractions (left).

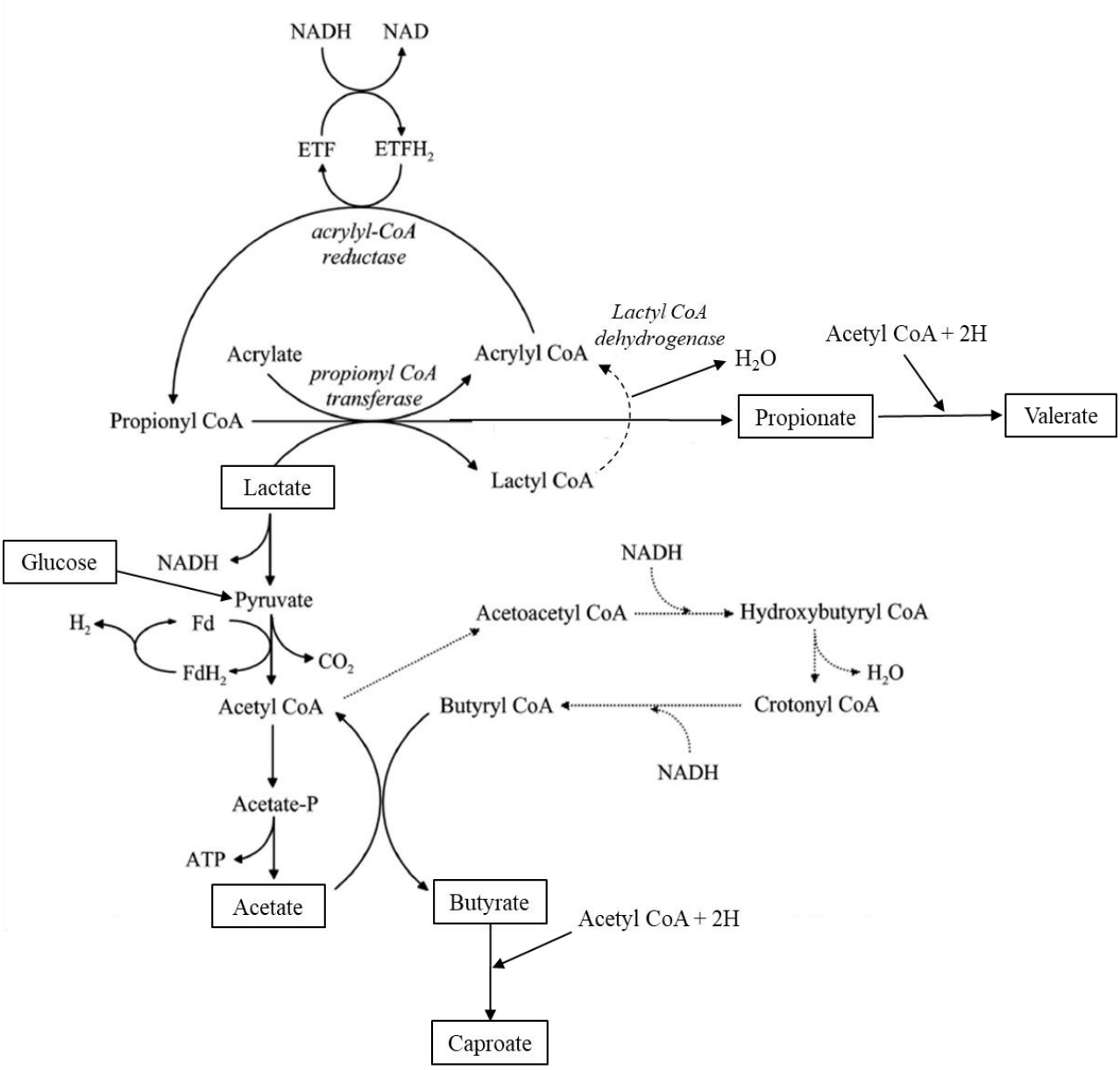

Figure 1.4 Proposed pathway for synthesis of propionate, acetate, butyrate, valerate and caproate from lactate or acrylate by *Megasphaera elsdenii* (adapted from Prabhu et al., 2012 and Weimer and Moen, 2013).

Chapter 2 - Investigation of effects of *Megasphaera elsdenii* strain NCIMB 41125 on growth performance and characteristics of cecal digesta in broiler chickens

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Abstract

A performance study was conducted to evaluate effects of *Megasphaera elsdenii* supplementation on growth performance and characteristics of cecal digesta in broiler chickens. Day-old male Cobb 500 broiler chicks (n = 2520) were separated into 72 pens of 35 birds each. Pens were blocked by location within a single barn and randomly assigned to 1 of 3 treatments. Treatment 1 consisted of a 0.2 mL oral gavage (OG) of *M. elsdenii* culture containing 1.97 x 10^9 CFU/mL of *M. elsdenii.* Treatment group 2 received an aerosolized mist (AM) of fresh *M. elsdenii* culture, applied to the body surface at a rate of approximately 1.7 mL/bird which contained 1.97 x 10^9 CFU/mL of *M. elsdenii*. Treatment 3 served as a negative control (C), having no direct contact with the bacterium. Feed and water were provided *ad libitum* via gravity feeders and sippers throughout the trial. Feed intake and pen weight data were collected at each feed change from starter to grower to finisher diets (d 16, 30 and 36). Cecal pH and volatile fatty acid (VFA) concentrations were evaluated each week (d 7, 14, 21, 28 and 35) using the cecal contents of 1 to 3 birds from each pen. At d 36, carcass data were collected from 5 birds from each pen. Growth performance, carcass weight, and mortality rate were unaffected by treatment ($P > 0.10$). Cecal pH was greater in C birds than in either OG or AM birds ($P < 0.01$). A treatment by day interaction was detected for VFA concentrations, with the cecal contents of OG birds containing greater acetate, propionate, butyrate, caproate and total VFA concentrations than that of C on d 14 ($P < 0.01$). Cecal acetate:propionate (A:P) ratio was greater in AM and OG birds than C on d 7 ($P < 0.03$). On d 21, acetate, propionate, butyrate and total VFA concentrations in the ceca of C birds were greater than either OG or AM birds. No differences in cecal VFA profile were observed across treatments after d 21 ($P > 0.30$). Administration of this probiotic culture had neither a detrimental nor beneficial effect on broiler health or growth

performance. *Megasphaera elsdenii* supplementation appeared to have the greatest effect on cecal VFA concentrations between d 14 and 21 following administration. After d 21, there appears to be no further effect of *M. elsdenii* on VFA profile in the broiler ceca. Decreased cecal pH observed in birds treated with this bacterium suggests potential for use as an acidifier, a commonly incorporated antibiotic alternative in poultry production.

Introduction

With the introduction of new federal regulations governing use of growth promoting antibiotics in food animal production due to concern of microbial antibiotic resistance, producers are presented with the challenge of finding alternative methods to maintain health of their flocks while maximizing feed efficiency (Dibner and Richards, 2005; FDA, 2015; Yirga, 2015). Growth promotion by antimicrobial compounds occurs through selective manipulation of populations of gastrointestinal (GI) microorganisms (Bedford, 2000; Hruby and Cowieson, 2006). Modulation of these microbial populations may improve gut health, prevent establishment of pathogenic organisms or treat clinical or subclinical infection (Landers, 2012). Several antibiotic alternatives have been investigated in poultry including exogenous enzymes, acidifiers, and probiotics.

Supplementation with exogenous enzymes alters microbial populations by increasing diet digestibility. This decreases the amount of substrate available to microbial populations within the large intestine by maximizing digestion in the small intestine (Bedford, 2000). Zanella et al. (1999) indicated that feed efficiency and weight gain in broiler chickens fed a corn and soybean meal based diet improved with the addition of a commercially available exogenous enzyme mixture, Avixyme® 1500, containing xylanase, protease and amylase. Furthermore, Zanella et al. (1999) demonstrated that supplementation with this enzyme mixture compensates for decreased provision of energy and nutrient availability without a reduction in performance.

Another common antibiotic alternative in poultry production is the use of organic acid compounds to serve as dietary acidifiers. Organic acids have been used extensively as feed preservatives, and their value as a modifier of the gut microflora has been well established (Ricke, 2003; Paul et al., 2007). Lückstädt et al. (2004) documented the effectiveness of an

acidifier blend to promote growth in broiler chickens. When administered a blended acidifier containing formic and propionic acid on a phyllo-silicate carrier with their diet, broilers exhibited greater body weight and average daily gain than controls. The influence of an acidifier to improve performance in broiler chickens was further evaluated in a study by Paul et al. (2007). Diets containing organic acid salts of either ammonium formate or calcium propionate increased small intestinal villous height when compared to control birds, indicative of improved gut health and absorptive capacity. This effect on epithelial morphology likely led to increased feed efficiency in birds receiving the acidifier compared to the control. Gut health was further affected by the provision of organic acid salts due to decreased coliform counts in the feed. Birds that received ammonium sulfate also possessed reduced *Escherichia coli* concentrations in their gastrointestinal tracts (GIT), which likely will result in decreased shedding of this pathogen in the feces, potentially lessening the transmission of disease. Acidifiers have been well established as a means of decreasing the transmission of pathogenic microorganisms in broiler production, most notably *Salmonella enterica* or *E. coli*, by inhibiting their colonization in the GIT (Mchan et al., 1992; Thompson and Hinton, 1997; Immerseel et al., 2005; Mountzouris et al., 2007; Paul et al., 2007).

Probiotics also have been used as an antibiotic alternative in poultry production. Administration of live cultures of beneficial microorganisms serve to stabilize the microbial populations and activity of the GIT, preventing a decline in microbial diversity and promoting population growth and activity of commensal microorganisms while inhibiting pathogen colonization (Chaucheyras-Durand and Durand, 2010). Stabilization of this activity results in improved performance and resistance to disease through several modes of action. These include suppression of pathogens, strengthening of the intestinal barrier and increasing nutrient retention

in the GIT (Patterson and Burkholder, 2003; Angel et al., 2005; Nava et al., 2005). There is ample evidence that supplementation with probiotic cultures containing several different lactate-producing bacterial (LAB) species limits the growth and fecal shedding of pathogenic bacteria like *S. enterica* (La Ragione and Woodward, 2003; Higgins et al., 2007a; Higgins et al., 2007b).

Increased retention of protein, Ca, P and N was observed in probiotic supplemented birds by Angel et al. (2005). Sen et al. (2012) demonstrated enhanced crude protein and energy digestibility in birds administered *Bacillus subtilis.* This probiotic also affected broiler gut morphology, increasing villus height and villus height to crypt depth ratio in the duodenum and ileum of treated birds. Increased villus height is associated with enhanced absorptive capacity of the small intestine which may result in improved feed efficiency and growth performance (Sen et al., 2012). Probiotic administration in the poultry industry is typically accomplished via application of an aerosolized spray over newly hatched chicks, followed by supplementation through drinking water (Ghadban, 2002). Administration via oral gavage has also proven to be an effective method of delivery for probiotic cultures; however, due to cost and labor constraints it is impractical in commercial production systems (Pascual et al., 1999; Nava et al., 2005). Delivery via the feed also is possible; however, this requires the microbial strain to be oxygen tolerant or somehow protected from the aerobic environment (Nava et al., 2005; Mountzouris et al., 2007). In the present study, *M. elsdenii* culture was administered via either oral gavage or spray application over the body surface of day old broiler chicks in a manner similar to that described by Corrier et al. (1994).

Megasphaera elsdenii is a novel microorganism with potential use in poultry production systems. This naturally occurring bacterium colonizes the lower GIT of birds and mammals (Hashizume et al., 2003; Tsukahara et al., 2006; Scupham et al., 2008). *Megasphaera elsdenii* is

a lactate-utilizing, Gram-negative coccus, regarded as an important bacterial species for the maintenance of normal gastrointestinal health and activity (Counotte et al., 1981). The present study was conducted to evaluate effects of *M. elsdenii* supplementation on broiler performance and cecal digesta parameters.

Materials and methods

All procedures followed in this study were approved by the Kansas State University Institutional Animal Care and Use Committee. This experiment was performed with 24 replicates of 3 treatments in a randomized complete block design. Treatments consisted of *M. elsdenii* strain NCIMB 41125 (MSBiotec, Wamego, Kansas) administered as either an oral gavage or an aerosolized mist applied to the body surface of birds, and a negative control (no direct contact with *M. elsdenii).* Pen was used as the experimental unit (n = 72), with each pen containing 35 birds at the onset of the experiment (2,520 birds total).

Facilities

Each of the 72 pens (2.43 m x 1.83 m) used in this experiment was located within a single building. The back and side barriers of each pen were lined with 4 mil polyethylene plastic sheeting to prevent direct contact between birds in adjacent pens, thus limiting the potential for cross contamination between treatments. Fresh pine shavings were used as bedding (approximately 8 cm depth) in each pen. Location within the barn was used as a blocking criteria to account for possible differences in ventilation and temperature. Treatments were randomly assigned to pens within each block. Groups of birds were processed by block, with experimental treatments being assigned randomly within each block.

Animals, diets, and treatments

Day-old male Cobb 500 broiler chicks were obtained from Cobb-Vantress in Siloam Springs, Arkansas, and transported to the Kansas State University Poultry Research Center in Manhattan, Kansas.

Administration of treatments

Prior to administration of *M. elsdenii,* 5-L foil bags of fresh culture were vigorously shaken to homogenize contents. Tygon tubing was used to connect a manually operated dosing device to the bag. The reservoir of the dosing device was repeatedly filled and dispensed to evacuate air. The contents were deemed free of ambient air when the culture, which contains an oxygen indicator, retained its normal color.

Twenty-four pens (35 birds/pen; 840 birds total) were dosed by oral gavage with 0.2 mL of a fresh probiotic culture containing 1.97×10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 using a Scorex Classic 173.05005 auto-filling syringe (Ecublens, Switzerland). Technicians restrained the birds by using the thumb and forefinger to hold the beak open while the contents of the syringe were discharged directly into the birds' oral cavities.

Twenty-four pens (35 birds/pen; 840 birds total) were dosed by aerosolized mist of a fresh culture containing 1.97×10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 applied by a pneumatic drenching device fitted with an atomizing tip. Birds were placed into a plastic tub (50 cm x 35 cm x 40 cm) and the culture was applied to their body surfaces as an aerosolized mist at a volume of 60 mL per pen (~1.7 mL/bird).

Twenty-four pens (35 birds/pen; 840 birds total) had no contact with *M. elsdenii* culture and served as controls. To prevent cross contamination with treated birds, control birds were handled only by designated personnel that had no contact with treated birds and placed in

designated carriers to be weighed and transferred to pens. In one case the birds were miscounted and pen 51 received 33 birds rather than 35 birds due to technician error.

Fresh water was offered *ad libitum* through sippers (6 sippers/pen) suspended from a water supply line. The sipper height was adjusted throughout the trial to accommodate growth of birds. Diets are shown in Table 2.1. All diets were fed in gravity feeders suspended in the center of each pen. Feed was added as needed to ensure *ad libitum* access throughout the duration of the study.

The starter diet was removed from the pens on d 16 of the study. Residual feed was weighed, removed from each feeder, and placed into numbered bins that corresponded with pen number. Feeders were refilled with the grower diet. This process was repeated on d 30 of the study, this time replacing the grower diet with the finisher diet. On d 36 the experiment was terminated and the residual finisher diet was weighed and recorded for each pen.

Total feed consumption per pen for each phase (starter, grower, and finisher) was calculated as:

Feed issued – feed recovered

Intake per bird per day was calculated as:

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

Pen weights

Pen weights were recorded at the end of each feeding period (starter, grower, finisher). At the end of the starter period (d 16), all birds in each pen were placed into a tub (50 cm x 35 cm x 40 cm) and weighed. The weight of the tub was subtracted from total weight to determine the weight of the birds in the pen. At the end of the grower period (d 30) all birds in each pen were placed into 2 tubs of equal weight (each 103 cm x 55 cm x 41 cm), weighed, and the weights

added together. The weight of each tub (taken prior to the birds being placed into them) was subtracted from total weight to determine the weight of the birds in the pen. At the end of the finisher period (d 36) all birds in each pen were placed into 2 tubs of equal weight (each 103 cm x 55 cm x 41 cm) and weighed. This time, the scale was tared with the tubs in place. The weight of the birds in each tub were then added to determine total pen weight. The scale was re-tared between pens to account for fecal accumulation. At each of the weighing periods, head count verification was performed as birds were placed into tubs.

Sample collections

Each week (d 7, 14, 21, 28 and 35), 1 to 3 birds were randomly selected from each pen and euthanized by cervical dislocation. Cecal contents (0.5 g) were collected and mixed with deionized water (2 mL) in a 20 mL HDPE scintillation vial (Fisher Sci.; 03-337-23B) using a vortex mixer (Scientific Industries Vortex-Genie 2 vortex mixer, Houston, TX). A portable pH meter (Thermo Scientific Orion 3-star portable pH meter, Waltham, MA), calibrated using pH 4.0, 7.0 and 10.0 standards was used to determine pH. Four parts of the cecal mixture were added to 1 part 25% w/v metaphosphoric acid solution and homogenized using a vortex mixer. The sample was then transferred into 2 microcentrifuge tubes in 1-mL aliquots and frozen at -18°C to await analysis of VFA.

On d 7 and 21 cecal contents were split into 2 aliquots. One aliquot was used for VFA analysis and prepared as explained above. The other (0.5 g) was placed directly into a separate 20-mL HDPE scintillation vial (Fisher Sci.; 03-337-23B) and frozen (-80°C) for quantification of bacterial numbers using quantitative, real-time PCR.

Laboratory analyses

Previously diluted and acidified cecal samples were thawed, homogenized using a vortex mixer and centrifuged at 24 x g for 18 min. The aqueous supernatant was transferred to gas chromatography vials. Volatile fatty acids were measured 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. Helium 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. Initial oven temperature was 80°C and temperature was increased at 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.

Carcass data

Birds were slaughtered at 5 wk of age to determine carcass weight. Feed was withheld approximately 4 h prior to slaughter. Five representative birds were selected from each pen and placed into catch boxes for transport to the processing area. The 5 birds were weighed by pen to determine live weight just prior to slaughter by stunning and exsanguination. The birds were bled for 2 min and then placed into a rotary scalder at 63°C for approximately 30 s. The birds were then transferred to a rotary drum mechanical plucker for 30 s for feather removal. The feet, head and shanks were removed and carcasses were eviscerated through an incision around the vent. Finally, carcasses were weighed by pen to determine hot carcass yield.

Statistical analyses

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

Results

PCR

Due to unforeseen difficulties with the DNA extraction step of the analysis, PCR was unsuccessful. Therefore, data is not available regarding the extent of microbial colonization of the broiler ceca by *M. elsdenii*.

Performance

Broilers demonstrated similar feed intake ($P = 0.68$), feed efficiency ($P = 0.92$; FE) and average daily gain ($P = 0.85$; ADG) across treatments (Table 2.2). Bird weights and mortalities also were unaffected by treatment ($P > 0.15$; Table 2.3). However, dressed yield was less for birds that received *M. elsdenii* as an oral gavage compared to that of birds that received *M. elsdenii* as an aerosolized mist ($P = 0.02$; Table 2.3).

Characteristics of cecal digesta

A treatment effect was detected for cecal pH (Table 2.4). Cecal pH was less in birds that received *M. elsdenii* by either mist or oral application, compared to that of control birds ($P < 0.01$). The mean cecal pH for C, AM, and OG treatment groups were 6.76, 6.63, and 6.60 (SEM = 0.046), respectively. Treatment effect in cecal pH likely was due in large part to pH differences between treatments on d 14 where pH in C birds was 6.67 compared to 6.25 and 6.11 in AM and OG birds, respectively.

A treatment by day interaction was detected for cecal acetate ($P < 0.01$), propionate ($P = 0.03$), butyrate ($P < 0.01$), acetate to propionate ratio ($P = 0.01$; A:P ratio), caproate ($P = 0.002$) and total VFA ($P < 0.01$) concentrations (Table 2.4). Acetate increased from d 7 to 14, peaking on d 14. Cecal contents of birds that received *M. elsdenii* as an oral gavage contained greater concentrations of acetate, butyrate and caproate than those of control birds on d 14 ($P < 0.01$). By d 21, acetate concentration decreased across all treatments; however, concentration of acetate in the ceca was greater in control birds when compared to birds treated with either aerosolized mist or oral gavage ($P < 0.01$). Propionate and butyrate concentrations also were greater in cecal contents of control birds than those of birds treated with *M. elsdenii* on d 21 ($P < 0.01$). Propionate concentration increased from d 7 to 21 across treatments and remained elevated until d 35, but did not differ between treatments on d 28 or d 35 ($P > 0.05$). The A:P ratio was greater in the cecal contents of birds treated with *M. elsdenii* compared to controls on d 7 ($P < 0.01$), with an A:P ratio of 31.96 (C), 41.33 (AM) and 42.03 (OG). On d 14 the cecal A: P ratio of birds treated with an aerosolized mist of *M. elsdenii* (40.44 mM) was greater than that of control birds (29.80 mM) or those that received an oral gavage of *M. elsdenii* (33.69; $P < 0.03$). Cecal A: P ratio was not different across treatments on d 21 to 35 ($P > 0.05$). Isobutyrate, valerate, isovalerate, isocaproate and heptanoate concentrations in cecal contents were not affected by treatment ($P > 0.10$). Total cecal VFA concentration was greater in orally gavaged birds compared to control birds on d 14 ($P < 0.001$). However, total VFA concentration was less ($P < 0.05$) in the cecal contents of birds that received *M. elsdenii* as an aerosolized mist or an oral gavage (64.90 mM and 64.82 mM, respectively) than that of controls (87.57 mM) on d 21. Total cecal VFA concentrations were similar across treatments for days 7, 28 and 35 ($P > 0.30$).

Discussion

In the current study, similarities in ADG and FE indicate that *M. elsdenii* administered to broiler chickens had neither a beneficial nor detrimental effect on performance. This contradicts data collected in 2 unpublished studies at Kansas State University, where FE was shown to improve during the first 21 d of production in broiler chickens dosed with *M. elsdenii*. Although there have been a number of researchers who have demonstrated improvement in growth characteristics and feed intake of broilers in response to probiotic supplementation in several studies (Timmerman et al., 2006; Nayebpor et al., 2007; Awad et al., 2009), efficacy of probiotics in poultry production is dependent upon the environment, external or internal stressors and stage of production. Probiotics tend to be most effective in conditions of environmental stress, such as severe temperature variation, poor hygiene or husbandry practices or disease challenge (Patterson and Burkholder, 2003; Timmerman et al., 2006).

Carcass weights also were unaffected by treatment, however, an unexpectedly greater dressed yield percentage of AM birds compared to OG was observed. A possible reason for the observed increase in dressing percentage may be increased butyrate production and absorption, which may have led to greater intestinal weight in supplemented animals, although in this study butyrate concentrations were similar in AM and OG birds. Butyrate serves as an energy source for colonic and cecal mucosa, which leads to epithelial proliferation and increased cecal weight (Sakata, 1986; Le Blay et al., 1999; Daly et al., 2012). This hypothesis is supported by Yoshida et al. (2009), who observed increased butyrate production, improved recovery from mucosal atrophy and increased colonic mucosal thickness in piglets supplemented with a combination of *M. elsdenii* and *L. plantarum* at weaning.

Mortalities were analogous across treatments, suggesting no ill effects of *M. elsdenii* on bird health. The bacterium does, however, alter fermentation in the distal GIT. Cecal pH was less for birds treated with *M. elsdenii* compared to controls ($P < 0.01$), likely due to VFA and organic acid production by the bacterium. Reduction in pH may indicate a potential use for *M. elsdenii* as an acidifier, which are commonly employed by the poultry industry as antibiotic alternatives. Organic acids such as formic acid, propionic acid and butyric acid have been used as acidifiers in poultry production, primarily to decrease GI colonization and fecal shedding of pathogenic microorganisms (Mchan et al., 1992; Thompson and Hinton, 1997; Ricke, 2003; Immerseel et al., 2005; Paul et al., 2007). Although Thompson and Hinton (1997) noted a decrease in *Salmonella* concentrations in response to the bactericidal activity of fumarate and propionate when these organic acids were added to the diet, this reduction was limited to the anterior GIT of treated chickens. While effective in the crop to reduce pathogen colonization, propionate and fumarate are largely absorbed prior to reaching the cecum, the primary site of *Salmonella* colonization (Xu et al., 1988; Hume et al., 1993; Immerseel et al., 2005). In order to reach the distal GIT, organic acids must be protected from prececal digestion. This typically is accomplished via encapsulation. Immerseel et al. (2005) observed decreased *S. enterica* colonization in the ceca after *Salmonella* challenge, as well as decreased fecal shedding and transmission of the pathogen when chickens were administered encapsulated butyrate with their feed. Addition of organic acid to feed in an unprotected form also resulted in decreased populations of lactate producing bacterial (LAB) in the anterior GIT (Thompson and Hinton, 1997). This decrease in LAB can cause dysbiosis throughout the remainder of the GIT. Lactate producing bacteria are often provided as a probiotic culture with the intention of improving performance and decreasing pathogen colonization by either competitive exclusion, or

production of lactate, a bactericidal compound (La Ragione and Woodward, 2003; Higgins et al., 2007a; Higgins et al., 2007b; Nayebpor et al., 2007).

In this trial, M. *elsdenii* apparently reached the ceca and established sufficient populations to alter fermentation, evidenced by differences in cecal VFA concentrations between treatments. A single dose method of probiotic administration was only effective for about 21 d following administration in this experiment. After d 21, no effect of treatment on VFA production was observed. It is likely that the effects of this probiotic may be extended or enhanced by more frequent administration in either feed or water (Thompson and Hinton, 1997; Pascual et al., 1999; Immerseel et al., 2005; Mountzouris et al., 2007). *Megasphaera elsdenii* appeared to be most effective in altering the cecal environment from around d 14 post-administration, indicated by decreased pH as well as increased cecal butyrate, acetate, caproate and total VFA concentrations in supplemented birds. It is also possible that efforts to control cross-contamination between treatments were not completely effective, and colonization of the ceca of control birds by *M. elsdenii* occurred. Unexpected colonization of *M. elsdenii* in the lower GIT of control birds may explain the lack of differences between treatments observed after d 21.

Megasphaera is known to produce butyrate from the fermentation of lactate or glucose (Counotte et al., 1981; Marounek et al., 1989; Slyter et al., 1997; Hashizume et al., 2003; Prabhu et al., 2012), which supports the findings of this trial at d 14. Increased caproate production, presumably due to saccharolytic and proteolytic activity of *M. elsdenii* also was observed in this study (Miura et al., 1980; Marounek et al., 1989; Shetty et al., 2013). Glucose and lactate also are fermented by *M. elsdenii* to produce acetate, which may account for the greater concentrations of this VFA seen in OG chickens (Miura et al., 1980; Marounek et al., 1989; Hino et al., 1993).

Cecal propionate concentrations did not increase in AM or OG birds over C birds; this may be due to the rapid rate of passage of digesta in chickens, which may not allow sufficient time for lactate to accumulate or be fermented by *M. elsdenii* to produce propionate (Liu et al., 2017). Branched-chain fatty acid production by deamination of amino acids has also been observed as a fermentation product of *M. esldenii* (Forsberg, 1978; Miura et al., 1980; Marounek et al., 1989), although increased branched-chain fatty acid concentrations were not observed in the current experiment.

Cecal VFA profile alone cannot be used to completely explain the differences observed between treatments in pH. Total VFA concentrations were greater on d 14 in OG birds compared to the control, however, differences in individual fatty acids were not substantial enough to account for the observed pH differences. Therefore, we postulate that production of other organic acids not measured in the present study, such as formate, are responsible for the observed decrease in pH. Supporting this, Marounek et al. (1989) and Shetty et al. (2013), observed formate production by *M. elsdenii* from the fermentation of glucose.

Immerseel et al. (2004b) reported that encapsulated butyrate will effectively reduce *Salmonella* colonization in the ceca; however, encapsulated propionate and formate seemed to have the opposite effect, increasing *S. enterica* colonization. This conflicts with the findings of McHan and Shotts (1991) and Paul et al. (2007), who observed decreased gastrointestinal pathogen colonization when broilers were provided formic or propionic acid in the feed. Increased caproate concentrations in the ceca may also have antipathogenic implications. In a study by Immerseel et al. (2004a), caproate inhibited growth of *S. enterica in vitro* and decreased colonization of the pathogen *in vivo*. In this trial, caproate production was increased in the first 14 d after administration of *M. elsdenii* by OG. Because *M. elsdenii* produces these

antipathogenic compounds, it would likely be beneficial to measure changes in *S. enterica* or *E. coli* colonization and fecal shedding in response to *M. elsdenii* supplementation. Although not evaluated in the current study, inhibition of pathogenic colonization and prevention of disease is the primary purpose of acidifier supplementation in poultry production. Paul et al. (2007) also reported increased intestinal villus height in response to organic acid salt supplementation. This contradicts the findings of Hernández et al. (2006), who observed no changes in jejunal epithelial morphology in response to formic acid supplementation. In future studies of *M. elsdenii* supplementation to poultry, intestinal epithelial samples should be taken to determine potential alterations in gut health and absorptive capacity.

Paul et al. (2007) observed increased ADG and FE as a result of feeding diets containing propionate or formate salts. However, in this study, no changes in feed conversion or growth performance were observed in response to supplementation with *M. elsdenii*. Hernandez et al. (2006) observed no effect on broiler performance in response to organic acid supplementation, however, the authors noted that organic acid addition to the feed slightly improved ileal digestibility of nutrients. Diebold and Eidelsburger (2006) indicated that the ability of organic acid or probiotic supplementation to alter the GI environment or improve growth performance in poultry is inconsistent and largely dependent upon the environment in which they are raised. Under ideal conditions of good hygiene and low stress, probiotic or acidifier addition to the diet are less effective (Patterson and Burkholder, 2003; Hernandez et al., 2006).

Conclusion

In summary, *M. elsdenii* did affect growth performance or mortality rates in broiler chickens. Consideration should be made of this bacterium's potential for use as an acidifier to reduce pH and increase organic acid production in the ceca. Supplementation with *M. elsdenii*

appears to be an effective method of increasing production of bactericidal compounds, such as butyrate or caproate in the distal GIT in addition to decreasing the pH of the ceca on d 14. It would likely be beneficial to focus future research on ascertaining this microorganisms' ability to improve gastrointestinal health and decrease pathogenic colonization and transmission in broiler chickens.

Limitations

One of the limitations of this study is the method of administration of the probiotic culture. While an effective means of delivery, administration of an oral gavage to broiler chickens, particularly in a commercial operation, is impractical and labor intensive. Administration via aerosolized mist offers a more efficient method of administration, however future studies should examine also delivery of this probiotic via either the feed or water to be more viable for commercial operations. Because PCR was unsuccessful, differences in colonization in the lower GIT of treated versus untreated birds were not evaluated. Therefore, the possibility of cross-contamination between treatments cannot be dismissed. Although efforts were made to eliminate potential for cross-contamination, it is possible that lack of differences between treatments after d 21 may be due to *M. elsdenii* colonization in the ceca and colon of control birds. Analysis of cecal characteristics should include organic acids such as formate or lactate in addition to VFA and pH analysis, to aid in understanding of factors involved in acidification of digesta. Collection of intestinal mucosal samples, to observe whether morphological changes occur in response to supplementation with *M. elsdenii*, would add to understanding of the effects of this bacterium. Digesta and feces also should be analyzed for the presence of pathogens to determine if *M. elsdenii* affects disease load or fecal transmission of pathogens.

Table 2.1 Composition of broiler experimental diets

	Dietary Phase[†]		
Ingredient	**Starter**	**Grower**	**Finisher**
Ground corn	55.26	59.74	65.06
Dehulled soybean meal, 47% CP	37.15	32.60	27.90
Soybean oil	3.10	3.35	3.10
Ground limestone	1.45	1.40	1.25
Salt	0.37	0.37	0.37
Monocalcium phosphate, 21%*	1.70	1.60	1.40
Sodium bicarbonate	0.22	0.19	0.17
Vitamin and mineral premix[ᵀ]	0.25	0.25	0.25
L-lysine hydrochloride	0.33	0.30	0.17
L-methionine	0.13	0.15	0.28
L-threonine	0.04	0.05	0.07

[†]Diets were pelleted through a 3-mm die, cooled, crumbled, and dispensed into paper sacks for storage until feeding. Diets were provided *ad libitum* within each dietary phase

*Biofos®, Mosaic Co., Plymouth MN

[ᵀ]Nutrablend poultry VTM premix, Neosho, MO

Table 2.2 Effects of *Megasphaera elsdenii* on broiler growth performance

					P-value	
Item*	**C[1]**	**AM[2]**	**OG[3]**	**SEM**	**Treatment ᵀ**	**Contrast ᵀᵀ**
Starter						
Feed intake, kg	38.8	39.1	39.2	0.43	0.69	0.40
Feed:gain	1.23	1.24	1.23	0.007	0.65	0.58
ADG, kg	31.6	31.6	31.9	0.31	0.57	0.53
Grower						
Feed intake, kg	142.0	142.4	141.6	0.86	0.67	0.97
Feed:gain	1.46	1.47	1.56	0.005	0.47	0.70
ADG, kg	97.3	97.1	97.2	0.76	0.97	0.80
Finisher						
Feed intake, kg	179.0	180.7	178.9	1.40	0.27	0.47
Feed:gain	2.19	2.17	2.19	0.043	0.91	0.88
ADG, kg	82.0	84.0	82.4	2.16	0.60	0.51
Overall						
Feed intake, kg	98.6	99.1	98.8	0.63	0.68	0.49
Feed:gain	1.55	1.55	1.54	0.006	0.92	0.95
ADG total, kg	63.8	64.0	64.0	0.54	0.85	0.57

[1] Birds had no direct contact with *M. elsdenii*
[2] Birds received *M. elsdenii* as an aerosolized mist applied to their body surfaces at a rate of ~ 1.7 ml/bird (1.97×10^9 CFU/mL)
[3] Birds received 0.2 ml *M. elsdenii* as an oral gavage (1.97×10^9 CFU/mL)
ᵀ Effect of treatment
ᵀᵀ Contrast of *M. elsdenii* vs. control

Table 2.3 Effects of *Megasphaera elsdenii* on bird weights, carcass characteristics, and mortalities

					P-value	
Item	**C[1]**	**AM[2]**	**OG[3]**	**SEM**	**Treatment [τ]**	**Contrast [ττ]**
Bird weight*						
d 1	40	40	40	0.2	0.48	0.23
d 7	157	155	159	2.2	0.39	0.90
d 14	437	448	443	5.9	0.34	0.20
d 16	559	557	561	3.3	0.71	0.90
d 21	1016	996	1028	12.5	0.18	0.79
d 28	1786	1784	1792	21.2	0.95	0.95
d 30	1948	1948	1950	10.5	0.98	0.93
d 35	2487	2493	2472	33.5	0.89	0.91
d 36	2550	2552	2553	14.5	0.99	0.91
Harvest weight	2580	2568	2591	21.0	0.69	1.00
Carcass weight	1833	1832	1832	15.1	1.00	0.96
Dressed yield, %	70.99^{AB}	71.38^{B}	70.68^{A}	0.220	0.02	0.84
Mortalities, %	3.34	3.35	1.95	0.851	0.41	0.50

*Bird weights reported in g

[1] Control birds had no direct contact with *M. elsdenii*

[2] Birds received *M. elsdenii* as an aerosolized mist applied to their body surfaces at a rate of ~ 1.7 ml/bird (1.97×10^9 CFU/mL)

[3] Birds received 0.2 ml *M. elsdenii* as an oral gavage (1.97×10^9 CFU/mL)

[τ] Effect of treatment

[ττ] Contrast of *M. elsdenii* vs. control

[A, B] Means within a row without a common superscript are different at $P \leq 0.05$

Table 2.4 Effects of *Megasphaera elsdenii* on broiler cecal pH and VFA concentrations

						P-value	
Item*	**Day**	**C[1]**	**AM[2]**	**OG[3]**	**SEM**	**Treatment ᵀ**	**Contrast ᵀᵀ**
	7	6.87$^{A\ a}$	6.85$^{A\ ac}$	6.81$^{A\ a}$			
	14	6.67$^{A\ a}$	6.25 $^{B\ b}$	6.11 $^{B\ b}$			
	21	6.15 $^{A\ b}$	6.12 $^{A\ b}$	6.25 $^{A\ b}$			
	28	6.83 $^{A\ a}$	6.81$^{A\ a}$	6.67 $^{A\ ac}$			
pH	35	7.26 $^{A\ c}$	7.10 $^{A\ c}$	7.15 $^{A\ d}$	0.103	T, D	0.01
	7	53.53 $^{A\ a}$	58.66 $^{A\ a}$	58.42 $^{A\ a}$			
	14	61.79 $^{A\ a}$	66.20 $^{AB\ a}$	74.00 $^{B\ b}$			
	21	61.48 $^{A\ b}$	47.66 $^{B\ b}$	46.40 $^{B\ c}$			
	28	40.42 $^{A\ bc}$	38.83 $^{A\ b}$	42.36 $^{A\ cd}$			
Acetate	35	39.13 $^{A\ bc}$	41.83 $^{A\ b}$	36.22 $^{A\ d}$	3.604	D, I	0.91
	7	1.82 $^{A\ a}$	1.48 $^{A\ a}$	1.52 $^{A\ a}$			
	14	2.35 $^{A\ ab}$	2.10 $^{A\ a}$	2.69 $^{A\ ab}$			
	21	6.36 $^{A\ c}$	4.01 $^{B\ b}$	3.63 $^{B\ bc}$			
	28	3.73 $^{A\ bd}$	4.08 $^{A\ b}$	4.21 $^{A\ c}$			
Propionate	35	4.58 $^{A\ d}$	5.88 $^{A\ c}$	5.96 $^{A\ d}$	0.608	D, I	0.51
	7	31.96 $^{A\ a}$	41.33 $^{B\ a}$	42.03 $^{B\ a}$			
	14	29.80 $^{A\ a}$	40.44 $^{B\ a}$	33.69 $^{A\ b}$			
	21	12.36 $^{A\ c}$	13.62$^{A\ bcd}$	13.57 $^{A\ c}$			
	28	13.25 $^{A\ c}$	12.96 $^{A\ cd}$	14.30 $^{A\ c}$			
A: P $^{\alpha}$	35	9.81 $^{A\ c}$	8.33 $^{A\ d}$	7.80 $^{A\ d}$	2.168	T, D, I	0.003
	7	5.60 $^{A\ a}$	5.54 $^{A\ a}$	5.73 $^{A\ a}$			
	14	9.45 $^{A\ b}$	10.95 $^{AB\ b}$	13.40 $^{B\ b}$			
	21	17.79 $^{A\ c}$	11.77 $^{B\ b}$	13.45 $^{B\ b}$			
	28	6.67 $^{A\ ab}$	7.02 $^{A\ ac}$	7.35 $^{A\ ac}$			
Butyrate	35	8.15 $^{A\ b}$	9.70 $^{A\ ab}$	8.40 $^{A\ ac}$	1.094	D, I	0.73
	7	0.39 $^{A\ a}$	0.36 $^{A\ a}$	0.34 $^{A\ a}$			
	14	0.37 $^{A\ a}$	0.35 $^{A\ a}$	0.45 $^{A\ a}$			
	21	0.38 $^{A\ a}$	0.17 $^{B\ b}$	0.15 $^{B\ b}$			
	28	0.05 $^{A\ b}$	0.00 $^{A\ c}$	0.00 $^{A\ b}$			
Isobutyrate	35	0.37 $^{A\ a}$	0.37 $^{A\ ad}$	0.34 $^{A\ a}$	0.055	D	0.04

Table 2.4 continued: Effects of *Megasphaera elsdenii* on broiler cecal pH and VFA concentrations

						P-value	
Item*	**Day**	**C[1]**	**AM[2]**	**OG[3]**	**SEM**	**Treatment ᵀ**	**Contrast ᵀᵀ**
	7	0.29 $^{A\,a}$	0.31 $^{A\,a}$	0.29 $^{A\,a}$			
	14	0.68 $^{A\,bc}$	0.71 $^{A\,b}$	0.90 $^{B\,b}$			
	21	1.13 $^{A\,c}$	0.91 $^{B\,b}$	0.96 $^{AB\,b}$			
	28	0.32 $^{A\,ac}$	0.28 $^{A\,c}$	0.34 $^{A\,a}$			
Valerate	35	0.63 $^{A\,b}$	0.79 $^{A\,b}$	0.68 $^{A\,c}$	0.078	D	0.89
	7	0.317 $^{A\,a}$	0.292 $^{A\,a}$	0.314 $^{A\,a}$			
	14	0.375 $^{A\,a}$	0.357 $^{A\,ab}$	0.505 $^{A\,b}$			
	21	0.419 $^{A\,a}$	0.396 $^{AB\,b}$	0.257 $^{B\,a}$			
	28	0.038 $^{A\,b}$	0.040 $^{A\,c}$	0.050 $^{A\,c}$			
Isovalerate	35	0.325 $^{A\,a}$	0.396 $^{A\,ab}$	0.355 $^{A\,ab}$	0.0592	D	0.96
	7	0.150 $^{A\,a}$	0.168 $^{A\,a}$	0.144 $^{A\,a}$			
	14	0.150 $^{A\,a}$	0.161 $^{A\,a}$	0.292 $^{B\,b}$			
	21	0.000 $^{A\,bc}$	0.000 $^{A\,b}$	0.001 $^{A\,c}$			
	28	0.000 $^{A\,c}$	0.000 $^{A\,b}$	0.001 $^{A\,c}$			
Caproate	35	0.000 $^{A\,c}$	0.000 $^{A\,b}$	0.001 $^{A\,c}$	0.0208	T, D, I	0.13
	7	0.125 $^{A\,a}$	0.116 $^{A\,a}$	0.142 $^{A\,a}$			
	14	0.085 $^{A\,b}$	0.101 $^{A\,a}$	0.078 $^{A\,b}$			
	21	0.000 $^{A\,c}$	0.001 $^{A\,b}$	0.001 $^{A\,c}$			
	28	0.000 $^{A\,c}$	0.001 $^{A\,b}$	0.001 $^{A\,c}$			
Isocaproate	35	0.000 $^{A\,c}$	0.001 $^{A\,b}$	0.001 $^{A\,c}$	0.0148	D	0.79
	7	0.177 $^{A\,a}$	0.186 $^{A\,a}$	0.146 $^{A\,a}$			
	14	0.104 $^{A\,b}$	0.082 $^{B\,b}$	0.103 $^{AB\,ac}$			
	21	0.000 $^{A\,c}$	0.002 $^{A\,c}$	0.003 $^{A\,b}$			
	28	0.000 $^{A\,c}$	0.001 $^{A\,c}$	0.003 $^{A\,b}$			
Heptanoate	35	0.000 $^{A\,c}$	0.001 $^{A\,c}$	0.053 $^{A\,bc}$	0.0239	D	0.88
	7	62.40 $^{A\,a}$	67.10 $^{A\,a}$	67.01 $^{A\,a}$			
	14	75.34 $^{A\,b}$	81.00 $^{AB\,b}$	92.39 $^{B\,b}$			
	21	87.57 $^{A\,b}$	64.90 $^{B\,a}$	64.82 $^{B\,ad}$			
	28	51.23 $^{A\,a}$	50.25 $^{A\,c}$	54.29 $^{A\,ac}$			
Total VFA	35	53.18 $^{A\,a}$	58.98 $^{A\,c}$	51.97 $^{A\,d}$	4.806	D, I	0.79

*All VFA concentrations reported in mM

[1] Control birds had no direct contact with *M. elsdenii*

[2] Birds received *M. elsdenii* as an aerosolized mist applied to their body surface at a rate of ~ 1.7 ml/bird (1.97×10^9 CFU/mL)

[3] Birds received 0.2 ml *M. elsdenii* as an oral gavage (1.97×10^9 CFU/mL)

$^{\alpha}$ Acetate to propionate ratio

ᵀ T = Effect of treatment; D = Effect of day of sampling; I = Interaction between treatment and day of sampling; $P \leq 0.05$

ᵀᵀ Contrast of *M. elsdenii* vs. Control

$^{A, B}$ Means within a row without a common superscript are different at $P \leq 0.05$

$^{a, b}$ Means within a column without a common superscript are different at $P \leq 0.05$

Chapter 3 - Effects of *Megasphaera elsdenii* NCIMB 41125 on growth performance and fecal characteristics of swine

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Abstract

A swine performance study was conducted to investigate effects of *Megasphaera elsdenii* supplementation on performance and fecal characteristics of peri-weaning piglets. Sow fecal characteristics and weight loss during lactation were also evaluated. Hermitage sows (n = 28) bred to a MaxPro boar were blocked by parity and randomly assigned to 1 of 4 treatments in a 2 x 2 factorial arrangement within a randomized complete block design. Factor 1 consisted of a 25-mL oral gavage containing 2 x 10^9 CFU/mL of *M. elsdenii* culture administered to sows just prior to farrowing or a negative control which received no probiotic culture. Factor 2 consisted of a negative control or 5-mL oral gavage containing 2 x 10^9 CFU/mL of *M. elsdenii* culture administered to litters of piglets 2 d after birth and at weaning (d 21). Fecal pH and volatile fatty acid (VFA) profiles were analyzed weekly from sows until weaning. Piglet feces were collected weekly for 5 wk after weaning. Piglet body weight and feed intake also were measured each week after weaning. *Megasphaera elsdenii* had no effect on litter size or sow weight loss during lactation ($P > 0.25$). No interaction effects between sow and piglet treatments were detected on overall piglet growth performance ($P > 0.10$); however, piglets dosed with *M. elsdenii* exhibited greater feed intake during the starter period upon weaning and introduction to the nursery ($P < 0.05$). Apparent interaction effect of sow treatment with piglet treatment on birth weight makes it difficult to evaluate growth performance of piglets ($P < 0.05$). Differences in growth may be attributed to treatment or to variation in initial body weights. *M. elsdenii* supplementation in sows tended to result in greater fecal concentrations of valerate, caproate and propionate ($P < 0.07$) when compared to controls. Piglets that received *M. elsdenii* directly exhibited increased fecal VFA concentrations over other treatment groups ($P < 0.05$). Butyrate, which was present in greater concentrations in feces of treated pigs, is important to prevent intestinal atrophy and

enhance recovery from stress-induced histopathies. Supplementation of sows and piglets with *M. elsdenii* affects fermentation in the large intestine and may increase feed intake immediately post-weaning, potentially increasing health and function of the gastrointestinal tract (GIT) during this critical period. In this trial, however, the effect of *M. elsdenii* supplementation on piglet growth was inconclusive.

Introduction

Piglet health and performance

Weaning and introduction to the nursery is an extremely stressful stage of commercial swine production. During the peri-weaning period, piglets undergo an abrupt change in both diet and environment which precipitates major shifts in microbial populations of the gastrointestinal tract (GIT; Konstantinov et al., 2004). These microbial shifts can result in dysbiosis, predisposing pigs to establishment and colonization by pathogens in the GIT. Microorganisms populating the porcine large intestine are responsible for fermentation of feed components which would be otherwise indigestible by the host. Volatile fatty acids (VFA) are produced by this microbial fermentation and are the primary anions of the large intestine. They are important in the absorption of sodium and water, as well as in controlling acidity of the cecum and colon. Commensal microbes of the large intestine also play roles in vitamin production and gut mucosal and immune development (Lallès et al., 2007; Chaucheyras-Durand and Durand, 2009).

Often, freshly weaned pigs will go between 1 and 3 d without eating following separation from their dams and introduction to the nursery (Brooks et al., 2001). This period of anorexia is associated with decreased villus height and crypt depth, which indicate reduced absorptive capacity in the small intestine (Verdonk et al., 2007). In fact, ramifications of weaning stress can include long-term impaired gut development and compromised barrier function of the intestinal epithelium (Lallès et al., 2007). All of these factors can result in a severe, and potentially lasting, reduction in growth, which causes economic loss for producers (Bontempo et al., 2006; Lallès et al., 2007). The consequences of weaning stress may be mitigated by increasing feed intake in baby pigs and balancing the microbial ecosystem of the gastrointestinal tract (GIT) during the nursery period by preventing a decline in microbial diversity while promoting growth and

activity of beneficial microorganisms (Mathew et al., 1998; Bontempo et al., 2006; Casey et al., 2007; Taras et al., 2007).

In the past, sub-therapeutic doses of antibiotics have been used to minimize pathogen colonization and diarrheal incidence in the post-weaning period (Bhandari et al., 2008). However, with the introduction of the 2017 Veterinary Feed Directive (VFD) in the United States, nonclinical administration of antibiotics in feed has been restricted. Therefore, it is important to find alternative methods of reducing weaning-induced diarrhea and anorexia. Probiotics have been studied as a potential alternative to antibiotics, as they may modulate microbial populations of the GIT during the post-weaning period. The most common microorganisms used as probiotics are lactate producing bacterial species (LAB) such as *Lactobacillus, Bacillus, Enterococcus* or *Pediococcus* spp. and have been shown to decrease pathogenic incidence and fecal shedding (Casey et al., 2007; Davis et al., 2007). One proposed method of reducing pathogen colonization was described by Mølbak et al. (2007), who reported that when pigs were fed a diet rich in fructans, *Bifidobacterium thermacidophilum* and *Megasphaera elsdenii* increased as a proportion of their total bacterial population. This diet was selected due to previously established ability to prevent swine dysentery. Researchers theorized that cross feeding between lactate-producing *B. thermacidophilum* and lactate utilizing *M. elsdenii* occurred in the colon. The authors further hypothesized that increased populations of *B. thermacidophilum* and *M. elsdenii* may play an important role in the inhibition of dysentery causing pathogen, *Brachyspira hyodysenteriae;* however, the mechanism by which this occurs has not yet been established.

Casey et al. (2007) observed decreased *Salmonella enterica* infection in piglets that had been supplemented with a mixed probiotic culture containing Lactobacilli and Pediococci spp.

following *S. enterica* challenge. Treated pigs exhibited decreased incidence, duration, and severity of diarrhea after the challenge as well as reduced fecal shedding of the pathogen. di Giancamillo et al. (2008), also using a *Pediococcus acidilactici* probiotic, reported improved villus height and crypt depth as well as increased enterocyte proliferation in response to supplementation with the bacterial culture. Pigs that received the probiotic demonstrated increased body weight and average daily gain (ADG) over their untreated counterparts. Proliferation of enterocytes and increased villus height and crypt depth is associated with healthy intestinal mucosa and the authors hypothesized that these piglets would experience improved pathogen resistance and nutrient absorption over the control animals. Other studies also have observed decreased pathogen colonization and diarrhea in response to probiotic administration during the weaning period. Scharek et al. (2005) reported decreased *E. coli* concentrations in response to supplementation with *Enterococcus faecium*; however, no changes in the commensal bacterial populations or intestinal morphology were detected. Taras et al. (2007) reported decreased diarrhea incidence and severity when pigs were provided *E. faecium* or *Bacillus cereus* at weaning. Although no differences in growth performance were reported with *E. faecium* supplementation, pigs that received *B. cereus* exhibited greater ADG and a lower feed to gain ratio (F:G) than controls. Conversely, Bhandari et al. (2008) who observed no change in feed intake or growth performance in response to supplementation with a *Bacillus* containing probiotic. However, decreased incidences of scours and mortality rates were observed in supplemented pigs following *E. coli* challenge. In a study by Mathew et al. (1998), yeast were fed to newly weaned piglets, which resulted in improved feed intake and growth. These findings were supported by Le Bon et al. (2010) who found that, when piglets were provided a probiotic containing yeast (*Saccharomyces cerevisiae*) and *P. acidolactici* at weaning, FE improved.

However, this improvement was not associated with morphological changes to the intestinal epithelium.

Sow health and performance

An important aspect of sow management is minimization of weight loss during lactation encouraging feed intake. Decreased intake, coupled with mobilization of body stores to meet energy requirements of lactation, may result in excessive weight loss during the parturition to weaning interval (Böhmer et al., 2006). Excessive weight loss during lactation often results in poor subsequent reproductive performance and is associated with lengthened wean-to-service interval, decreased subsequent litter size and poor farrowing rates, especially in parity 1 sows (Thaker and Bilkei, 2005). Probiotic intervention to prevent this "starvation sterility" may serve the dual purpose in sows of increasing feed intake, and therefore, energy availability, and stabilizing the microbiota of the GIT to prevent disease (Böhmer et al., 2006).

Alexopoulos et al. (2004) reported increased feed consumption in lactating sows treated with a probiotic containing *Bacillus* spp. Böhmer et al. (2006) examined the effect of supplementation of sows with a different probiotic, and reported increased feed intake and decreased morbidity in sows treated with *Enterococcus faecium* as a feed additive during gestation, compared to controls. In both studies, sows exhibited greater body weights at the end of lactation and more piglets born alive per litter. Their litters also gained more weight, exhibited decreased morbidity rates and had greater weaning weights than piglets of control sows. *Enterococcus faecium* and *Bacillus* spp. increased feed intake in treated sows, especially gilts. Although an explanation is not well-established, it may be that intake increases due to improved overall gut health and decreased gastrointestinal (GI) upset, thereby stimulating appetite. Böhmer

et al. (2006) also reported decreased body temperature in treated sows, which was attributed to stimulation of the immune system and enhanced immune performance.

An *in vitro* model of the porcine cecum with added *M. elsdenii* probiotic culture, revealed an increase in production of 4- and 5-carbon fatty acids, specifically butyrate and valerate, over the control (Tsukahara et al., 2006). *Megasphaera elsdenii* appears to be especially effective in the presence of LAB, in this case, *L. acidophilus,* due to cross-feeding which occurs between the two microorganisms. Lactate produced by *L. acidophilus* is fermented by *M. elsdenii* to produce VFA such as propionate or butyrate. Fermentation of lactate by *M. elsdenii* prevents lactate accumulation, thereby preventing feedback inhibition of *L. acidophilus* activity (Tsukahara et al., 2006). Increased valerate production observed in this experiment is likely due to metabolism of amino acids or propionate by *M. elsdenii* (Weimer and Moen, 2013). Butyrate is the preferred energy source for colonocytes and is known to stimulate differentiation and proliferation of the epithelium of the large intestine (Bergman, 1990; Daly et al., 2001; Tsukahara et al., 2006). Butyrate also helps prevent mucosal atrophy, stimulate mucin secretion and aid in strengthening the barrier function of the intestinal mucosa (Hamer et al., 2008). Increasing production of butyrate may improve intestinal health, thereby decreasing pathogenic infection and enhancing nutrient absorption in both sows and piglets during the farrowing to weaning interval and the post-weaning period respectively. This study was designed to determine effects of supplementation of sows and piglets with *M. elsdenii* on growth performance and fecal characteristics during the suckling and peri-weaning period.

Materials and methods

All procedures followed in this study were approved by the Kansas State University Institutional Animal Care and Use Committee.

Facilities

This experiment was performed at Midwest Veterinary Services Inc., Oakland, NE. Sows were housed in 1.52 x 2.13 m farrowing crates within a single barn from 1 to 3 d prior to their expected farrowing dates until weaning at approximately d 21 post-farrowing. Piglets were moved into a separate nursery after weaning and separated into pens according to litter. Pens (2.44 x 2.44 m) were located within a single barn and all spatial requirements established by the "Guide for the Care and Use of Agricultural Animals in Research and Training" (Salek-Johnson et al., 2010) were either met or exceeded for both sows and piglets.

Animals, diets, and treatments

Twenty-eight Hermitage sows bred to a MaxPro boar were blocked by parity and randomly assigned to 1 of 4 treatments in a randomized complete block design with a 2 x 2 factorial arrangement of treatments. Factor 1 consisted of a negative control which had no direct contact with the probiotic culture, or an oral gavage with *M. elsdenii* NCIMB 41125 (MSBiotec, Wamego, Kansas) administered to sows. Factor 2 consisted of a negative control or oral gavage containing *M. elsdenii* administered to litters of piglets 48 h after birth and again at weaning. Experimental unit was sow and her litter. Treatment assignments and the number of pigs in each litter are summarized in Table 3.1. Treatment groups consisted of Nn, where neither sows nor piglets received *M. elsdenii* culture, Ny where piglets but not sows received *M. elsdenii* culture, Yn where sows but not piglets received *M. elsdenii* and Yy, where both sows and piglets received *M. elsdenii.* Piglets were cross-fostered within treatments to equalize litter size.

Administration of treatments

Prior to administration of oral gavage, 5-L bags of fresh *M. elsdenii* culture were vigorously shaken to homogenize the contents. Tygon tubing was used to attach a manually operated dosing device to the bag. The reservoir of this device was filled and its contents subsequently discarded into a waste container. Approximately 100 to 200 mL of the fresh culture was discarded to ensure the device and tubing would be free of oxygen.

One to 3 d before farrowing, 14 sows were restrained with a snare and dosed by oral gavage with 25 mL of fresh culture containing 2 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125. The remaining 14 sows had no direct exposure to the probiotic and served as controls. All sows were then placed into farrowing crates (1.52 m x 2.13 m) within the same barn.

Forty-eight h after farrowing, piglets in the litters from 7 control sows and 7 orally dosed sows were restrained by hand and dosed by oral gavage with 5 mL of fresh probiotic culture containing 2 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125. The piglets in the remaining 14 litters (7 from control sows and 7 from oral gavage sows) received no probiotic.

At weaning, approximately 21 d post-farrowing, previously treated litters were again restrained by hand and given a second 5-mL oral gavage of 2 x 10^9 CFU/mL of *Megasphaera elsdenii* strain NCIMB 41125. Piglets were subsequently weaned and placed by litters into pens (2.44 m x 2.44 m) located within the same barn.

Feeding and watering

Fresh water was provided *ad libitum* through stainless steel nipple waterers. Diets provided during this experiment (gestation, lactation, and weaning) are shown in Table 3.2. Sows were limit fed during gestation to meet NRC requirements. After parturition, feed was increased by 4.4 kg/d until an *ad libitum* intake was achieved. Daily feed consumption was recorded during

both gestation and lactation. Non-medicated milk replacer was added to pens 2, 14, 17, 22, and 28 to supplement nutrition to large litters during the suckling period.

Litters of piglets were weaned on approximately d 21 post-farrowing. Upon introduction to the nursery, they were placed on a common nursery diet (Table 3.2) and fed via a stainless-steel gravity feeder. Feed was replenished as needed, and weight of the added feed recorded to ensure *ad libitum* access throughout the study. Weights of residual feed were recorded on d 28, 35, 42 and 49 of the experiment. Upon termination of the experiment, unconsumed feed was removed from each feeder and weights of the residual feed recorded.

Total feed consumption per pen was calculated as:

Feed added to feeder – feed recovered from feeder

Average daily feed intake (ADFI) was calculated as:

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

Individual sow weights were taken as they were placed into farrowing crates and once again at weaning. Piglets were weighed individually on d 2 (neonatal processing), 21 (weaning), 28, 35, 42, and 49 (weekly) of the experiment. Head counts in each pen were verified at each weighing.

Sample collection

Fecal samples were collected by rectal palpation using nitrile exam gloves, as sows were placed into farrowing crates. Samples were again collected on d 7, 14, and 21 post-farrowing. Target sample weight was at least 5 g per animal. Samples were placed into 50-mL Falcon conical centrifuge tubes (Corning Inc. 352070; Corning, NY), immediately snap frozen by immersing in a liquid nitrogen bath for 30 sec, then stored at -80°C.

For piglets, fecal samples were collected rectally from at least ½ of the population in each litter using nitrile exam gloves on d 21, 28, 35, 42, and 49 after farrowing. Target sample weight was a minimum of 5 g per animal. Samples were placed into 50-mL Falcon conical centrifuge tubes (Corning Inc. 352070; Corning, NY), immediately snap frozen by immersing in a liquid nitrogen bath for 30 seconds, and stored at -80°C.

One piglet per litter was selected at random on day 28 (day 7 post weaning) and euthanized using a barbiturate solution (Fatal Plus, Vortech Pharmaceuticals, Ltd., Dearborn, MI). Total cecal contents were placed into 50-mL Falcon conical centrifuge tubes (Corning Inc. 352070; Corning, NY), snap frozen by in a liquid nitrogen bath for 30 seconds, then stored at -80°C to await PCR analysis. Several pigs were treated with antibiotics for respiratory infection or *E. coli* over the course of this study. Fecal samples of treated pigs were not included in the analyses.

Laboratory analyses

Feces were thawed and then mixed with water using a vortex mixer (Scientific Industries Vortex-Genie 2 vortex mixer, Houston, TX; 0.5 g feces in 4 mL deionized water) and pH was taken using a benchtop pH meter (Orion 3-star portable pH meter, Thermo Scientific Waltham, MA). Four parts of the resulting mixture were combined with 1 part of a 25% w/v metaphosphoric acid solution and homogenized using a vortex mixer. The solution was then transferred into 2 microcentrifuge tubes (approximately 1 mL each) and frozen. After being frozen for at least 24 h, samples were thawed, homogenized with a vortex mixer, and centrifuged at 17,000 x g for 18 min. The top lipid layer was discarded, and the aqueous supernatant beneath was removed by pipette and transferred to gas chromatography vials.

Volatile fatty acids were quantified using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Nukol™ capillary column (30 m x 0.53 mm x 0.5 mm film thickness; Sigma Aldrich, St. Louis, MO) and flame ionization detector. Helium was used as the carrier gas at a flow rate of 22 cm/second, with a 1-µL split injection and split flow of 50:1. Initial oven temperature was 80°C, and temperature was increased at 10°C/minute to a final temperature of 220°C. Injection and detector temperatures were 250°C. Volatile fatty acids were quantified by comparing to known standards (Supelco Volatile Fatty Acid Mix; Sigma-Aldrich, St. Louis, MO) containing acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate, caproate, and heptanoate.

Statistical analyses

Data were analyzed using the Mixed procedure of SAS Version 9.4. The model included fixed effect of treatment, random effect of replicate, with sow or litter as the experimental unit. Interaction between sow treatment and piglet treatment was assessed for piglet growth and performance data. Interaction between treatment of sows, treatment of piglets and day was evaluated for fecal parameters. Interaction effects of sow treatment by piglet treatment were also considered, as well as sow treatment by day and piglet treatment by day and main effects of sow treatment and piglet treatment. Significance was declared at $P \leq 0.05$. 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

PCR

Due to unforeseen difficulties with the DNA extraction step of the analysis, PCR was unsuccessful. Therefore, quantification of *M. elsdenii* populations in porcine feces was not accomplished.

Performance

Sows exhibited no differences in litter size or lactational weight loss in response to *M. elsdenii* supplementation (Table 3.3; $P > 0.25$). Piglet growth performance, however, was affected by both treatment of the sows with *M. elsdenii* and treatment of the litters. A sow treatment by piglet treatment interaction was detected for birth weight ($P < 0.05$), but not for any other growth parameter ($P > 0.10$). Piglet birth weight was greater in Ny and Yy litters compared to Nn or Yn litters (1.79 kg and 1.60 kg compared to 1.38 kg and 1.32 kg respectively; Table 3.4; $P < 0.001$). Sows that did not receive the probiotic culture also weaned heavier piglets (6.55 kg compared to 5.80 kg) and their litters exhibited greater ADG in the nursery compared to treated sows' values, with the greatest weaning weight observed in Ny pigs ($P < 0.01$). Weaning weights of Yy piglets were similar to Nn piglets (6.11 kg compared to 6.41 kg; $P > 0.10$), with minimum weaning weight being observed in Yn pigs (5.50 kg; $P < 0.05$). Nursery ADG was similar across treatments Nn, Ny and Yy, while Yn nursery ADG was less than Nn or Ny ($P < 0.05$) but similar to Ny ($P = 0.30$). Pigs in the Ny treatment group were consistently heavier than piglets in the other groups in the post-weaning period when weighed each week, from weaning to d 28 ($P < 0.001$). Treated piglets were heavier than untreated piglets in each of the 4 wk following weaning; while those in the Yn treatment group tended to exhibit the lightest body weights ($P < 0.05$).

During the starter period, the 7 days following weaning, Yy piglets experienced less depression of feed intake than Nn or Yn litters (Table 3.4; $P < 0.03$), while Ny litters had similar feed intake to all other treatment groups ($P > 0.10$). Increased ADFI during this period correlated with improved ADG during the first week post-weaning in piglets that received the probiotic (Ny and Yy; both 0.09 kg/d) compared to those that did not (NN and YN; 0.05 kg/d and 0.06 kg/d respectively; $P < 0.05$). Average daily feed intakes during the grower phase and overall post-weaning were unaffected by treating either the sows or piglets with *M. elsdenii* ($P > 0.18$). Average daily gain during the grower period (wk 2 through 4) and total post-weaning ADG were less in the Yn litters than other treatment groups and greater in Ny litters than either Yn or Yy litters ($P < 0.05$); however, total post-weaning ADG of Yn litters tended to be less than those of Nn or Ny litters (0.31 kg/d compared to 0.34 kg/d or 0.36 kg/d respectively; $P < 0.05$), but similar to those of Yy litters (0.33 kg/d; $P > 0.10$). The overall ADG of the nursery period combined with the post-weaning period was greater in Ny piglets (0.31 kg/d) compared to those of Yn and Yy (0.28 or 0.29 kg/d; $P < 0.05$). Overall ADG of control litters (Nn; 0.30 kg/d) were similar to those of the other treatment groups ($P > 0.10$). Feed efficiency could not be calculated as feed intake was measured by pen, while body weights were collected individually and mortalities not weighed. Mortality percentage was unaffected by treatment (Table 3.4; $P > 0.40$).

Fecal characteristics

Fecal VFA profile in sows were affected by supplementation with *M. elsdenii* (Table 3.5). A trend for sow treatment by day interaction was detected for isobutyrate ($P < 0.10$), while a trend for main effect of treatment was observed for propionate, valerate and caproate ($P < 0.10$). Total fecal VFA concentrations tended to be greater in sows that received *M. elsdenii* (112.67 mM) than controls (90.10 mM) on d 7 ($P < 0.10$). Fecal propionate, butyrate and

isovalerate concentrations of treated sows were elevated above controls on d 7 ($P < 0.05$). Isobutyrate concentrations also tended to be greater on d 7 in sows that received the probiotic compared to those in the control group ($P < 0.10$). On the day treatment was administered, caproate concentrations were greater in sows belonging to the treatment group than the controls ($P < 0.05$). Acetate, valerate, isocaproate, and heptanoate concentrations were not different among treatments on any day fecal VFA concentrations were analyzed ($P > 0.10$).

Piglet fecal characteristics also were affected by treatment of either sows or piglets with *M. elsdenii* culture (Table 3.6). A sow treatment by piglet treatment by day interaction effect was detected for isobutyrate and isovalerate concentrations ($P \leq 0.05$) with a trend noted in propionate concentrations ($P = 0.10$). A sow treatment by piglet treatment interaction effect was noted for fecal pH ($P < 0.02$), while a sow treatment by piglet treatment trend was detected for fecal butyrate concentrations ($P < 0.06$). Fecal pH was less on d 0, the day of weaning, in the Nn treatment group than Ny, Yn or Yy ($P < 0.05$), but did not differ among treatments on d 7, 14 or 28. However, on d 21, fecal pH was greater in Yn pigs than Nn (6.63 compared to 6.32; $P < 0.02$) although it was similar to the other treatment groups ($P > 0.05$). Both sow treatment by day and piglet treatment by day interaction effects were detected in total VFA and acetate concentrations ($P < 0.01$). Total fecal VFA, acetate, or propionate concentrations did not differ between treatments on d 0 ($P > 0.05$). Butyrate concentrations in the feces were greater in Nn pigs on d 0 than those of the other treatment groups ($P < 0.05$), and did not differ between Ny, Yn or Yy ($P > 0.05$). Fecal isobutyrate concentrations were less in Yy pigs on d 0 compared to Nn, Ny and Yn ($P < 0.05$), while the remaining treatment groups did not differ in isobutyrate concentrations on this day ($P > 0.05$). Isovalerate and caproate concentrations were greatest in

the feces of Nn pigs compared to other treatment groups on d 0 ($P < 0.05$). Valerate was greater in Nn, and Yn pigs ($P < 0.05$) than Ny or Yy pigs on d 0.

Fecal acetate concentrations were elevated in Ny and Yy pigs over Nn on d 7 ($P < 0.05$), while concentrations of acetate in feces of the Yn treatment group were similar to those of Nn ($P > 0.05$). Propionate, isovalerate and valerate concentrations were less in Nn pigs than Ny, Yn or Yy pigs on d 7 ($P < 0.05$). Greater fecal valerate concentrations were observed on d 7 in Yy pigs compared to piglets in other treatment groups ($P < 0.05$). Isobutyrate and butyrate concentrations were greater in Yy and Ny pigs than Nn ($P < 0.05$), while Yn concentrations of these fatty acids was similar to Nn and Yy on d 7 ($P > 0.05$). Fecal caproate concentrations on d 7 were greater in Yy pigs (0.249 mM) compared to those of Ny pigs (0.024 mM; $P < 0.05$), although it did not differ from pigs in either Nn or Yn treatment groups ($P > 0.05$). Caproate concentrations remained analogous among treatments from d 14 through 28.

On d 14, fecal concentrations of acetate, propionate, butyrate, isobutyrate and isovalerate were less in Nn pigs than in Ny, Yn or Yy pigs ($P < 0.05$). Greater concentrations of fecal acetate, propionate and total VFA were observed in the Yy treatment group compared to Nn or Yn ($P < 0.05$), although they were not different from Ny ($P > 0.05$). Valerate was greater in feces of Ny and Yy pigs than Nn ($P < 0.05$), although Yn fecal valerate concentrations were the same as Nn, Ny and Yy ($P > 0.05$).

On d 21 few treatment differences were detected. Fecal acetate and total VFA concentrations were greater in Yy pigs than the other treatment groups. ($P < 0.05$). Valerate, butyrate, isobutyrate, and isovalerate concentrations in the feces were elevated above those of Ny pigs in Yy pigs on d 21 ($P < 0.05$), however concentrations of these VFA were similar in Yy, Nn and Yn pigs on this day. By 28 d post-weaning, total fecal VFA concentration and concentrations

of each individual VFA, except caproate, were greater in Ny and Yy treatment groups that Nn or Yn ($P < 0.05$).

Discussion

Excessive loss of body mass associated with lactation in sows may be detrimental to their future reproductive performances (Thaker and Bilkei, 2005; Böhmer et al., 2006). As such, it is important to minimize weight loss during this crucial period. The present study is the first to examine the effect of *M. elsdenii* on sow performance. In this study, supplementation of sows with *M. elsdenii* had no effect on lactational weight loss. This finding is in contrast to the observations of Jorggensen and Hansen (2006), who reported a reduction in lactational weight loss and increased ADFI in sows supplemented with a probiotic containing LAB, *Bacillus subtilus* and *B. licheniformis.* Böhmer et al. (2006) and Alexopoulos et al. (2004) also reported increased ADFI and minimization of weight loss when sows were supplemented with LAB-containing probiotics prior to farrowing. Litter size was expectedly unaffected by treatment, as administration occurred only 1 to 3 days prior to farrowing, too late in gestation to have any bearing on litter size or development.

The shift from nursing to a solid diet at weaning is a stressful event often associated with periods of anorexia and growth depression in piglets due to the abrupt change in both diet and environment (Brooks et al., 2001; Verdonk et al., 2007). Weaning also is correlated with diarrhea and damage to the intestinal mucosa which affects the absorptive capacity of the GIT. Decreased intestinal absorption can be attributed to decreased brush-border enzyme activity, decreased villus height and dysbiosis, all of which have been reported consequences of the physiological stress associated with weaning (Lallès et al., 2007). Anorexia in the days following weaning causes atrophy of the intestinal mucosa and weakens the barrier function of the intestinal

epithelium (Spreeuwenberg et al., 2001; Verdonk et al., 2007). Thus, stimulation of feed intake during the first week after weaning is important to manage gut health and improve subsequent growth performance (Lallès et al., 2007). Kelly et al. (1991) hypothesized that increasing feed intake during the immediate post-weaning period may increase secretion of the trophic hormone enteroglucagon. This results in enhancement of gut function and absorptive capacity, thus improving health and performance of the pig. In the current study, supplementation of piglets with *M. elsdenii* at birth and at weaning appears to be an effective means of increasing ADFI during the starter period, the critical first week after pigs are weaned and moved into the nursery. Average daily gain during the starter period also was improved in treated litters which received *M. elsdenii* directly via oral gavage, likely due to greater ADFI in pigs that received the probiotic supplement compared to the control. This improved growth performance was maintained through the post-weaning period.

Unexpected differences between treatment groups in piglet birthweight were observed; where treated piglets exhibited greater initial body weight than untreated piglets. This cannot be an effect of treatment as piglets were not administered the probiotic until after they were weighed and processed, approximately 48 h after birth, and sows received the probiotic culture between 1 and 3 days prior to farrowing. This phenomenon likely affected the growth data obtained in this study, due to differences in the piglets' planes of growth as influenced by their initial body weight. Quiniou et al. (2002) conducted a survey of almost 1000 litters of piglets and found that piglets born at greater birth weights have lower mortality rates and perform better than their lighter weight counterparts in each subsequent stage of growth. Indeed, in this study, piglets that began at a greater birth weight tended to maintain greater performance than the other treatment groups throughout the trial. Piglets in the Ny treatment group had the greatest initial

body weight and exhibited increased growth performance above other treatment groups through wk 4 post-weaning. Piglets in the Yy and Nn treatment groups performed similarly each week that piglets were weighed. Growth performance of Yn pigs was poorer than that of the other groups, however these pigs' initial body weights were less than those of pigs in the Ny or Yy treatment groups. Differences in initial body weight that cannot be attributed to treatment make it difficult to draw conclusions regarding the effect of *M. elsdenii* on growth performance of piglets in the suckling and peri-weaning period. Although differences were observed in piglet birth weights between treatments, no differences in mortality rates were detected. This is in contrast with Fix et al. (2010) who reported that as piglet birth weight decreases, survival rate to weaning and in the nursery also decreases. Differences in early mortality were likely not observed because average birth weights in each treatment group were above the 1.11 kg threshold established by Feldpausch et al. (2016) as having the greatest risk of peri-weaning mortality.

Volatile fatty acid production in the large intestine accounts for about 20% of the energy requirements of the pig (Imoto and Namioka, 1978; Rerat et al., 1987). Increasing VFA production in the cecum and colon should aid recently weaned pigs and lactating sows in meeting their energy requirements during periods of physiological and nutritional challenge. An increase in total fecal VFA was observed on d 7 post farrowing in sows. After d 7 however, VFA production between treatments remained similar. Total VFA production in weaned pigs increased when pigs received *M. elsdenii,* whether indirectly through the sow or directly via oral gavage. While fecal VFA concentrations reflect both production and absorption in the large intestine, there is no reason to believe absorption of VFA was altered in this study. Therefore, based on these data, sows and piglets receiving *M. elsdenii* may be assumed to have greater energy availability due to increased VFA production, as reflected in fecal VFA concentrations.

In this experiment, growth performance was not affected by the observed increase in total VFA. Supplementation of both sows and piglets with *M. elsdenii* appears to be most effective in augmenting microbial fermentation in the piglet large intestine. Supplementation of piglets directly, via oral gavage, appears to be effective for a longer period of time than simply supplementing sows and relying on maternal transfer of microbes to the litter, as the effects of sow supplementation without supplementation of piglets are no longer different from controls by d 28.

Administration of *M. elsdenii* affected microbial fermentation in the large intestine of both sows and piglets. Although sow fecal pH was not affected by treatment, alterations in VFA profile of the feces occurred in response to oral supplementation with the bacterial culture. Increased fecal propionate observed in both treated sows and piglets in the weeks following weaning may be attributed to the fermentation of lactate into propionate via the acrylate pathway which is characteristic of *M. elsdenii* (Prabhu et al., 2012). Increased concentrations of branched-chain fatty acids, isovalerate and isobutyrate, may be attributed to supplementation of sows and piglets with *M. elsdenii.* This LUB is able to ferment branched-chain amino acids to produce branched-chain fatty acids (Forsberg, 1978; Marounek et al., 1989). Branched-chain fatty acids serve as growth factors for cellulolytic bacteria which predominately produce acetate from the fermentation of fiber (Miura et al., 1980; Zhang et al., 2013). Increased acetate production is indicative of improved microbial balance in the large intestine as *M. elsdenii* competes with LAB for sugars, stabilizes pH, limits lactate production, and allows fibrolytic bacteria to maintain consistent populations (Daly et al., 2001; Zhang et al., 2013). Fecal caproate concentrations were greater in sows that received *M. elsdenii* than those that did not on d 0. This observation is not a function of treatment and maybe contributed to differences in feed intake between sows or

inherent differences in fermentation between individuals. Greater caproate concentration observed at weaning in Nn pigs contradicts the expected increase in caproate concentration resulting from supplementation of sows with *M. elsdenii*, as caproate is a product of glucose fermentation by the bacterium (Marounek et al., 1989; Shetty et al., 2013).

Butyrate can be produced by *M. elsdenii* from the fermentation of either glucose or lactate (Marounek et al., 1989; Hino and Kuroda, 1993; Kung Jr. and Hession, 1995). This was supported by the present study as fecal butyrate concentrations were greater in piglets that received *M. elsdenii* either directly or indirectly compared to Nn animals. As butyrate is the preferred energy source for colonic and cecal mucosa, increased butyrate production often is associated with enhancement of intestinal health. Tsukahara et al. (2006) found that increasing butyrate production in the large intestine promoted proliferation and differentiation of colonic epithelial cells. Crypt depth was also greater, which indicates increased absorptive and secretory capacity of the GIT, suggesting improved digestive function (Pluske et al., 1997). Greater numbers of mucin-containing cells also were observed in pigs' GIT in response to increased butyrate production. Mucin serves as a protective coating of the GIT, selectively allowing passage of nutrients and excluding pathogens while protecting against mechanical damage (Bansil and Turner, 2006; Zuo et al., 2015).

Following damage to the mucosa, butyrate has been shown to stimulate regeneration of intestinal epithelial cells and reduce inflammation (Wächtershäuser and Stein, 2000; Brouns et al., 2002). When infused into the colon of rats, butyrate had a regenerative effect in the large intestine; and when combined with an infusion of acetate and propionate, the trophic effect extended to both the small and large intestines (Kripke et al., 1989). By increasing production of butyrate, *M. elsdenii* may prevent or minimize intestinal atrophy associated with weaning. This

will enhance intestinal integrity thereby decreasing susceptibility to disease and increasing nutrient absorption. Butyrate also has selective antibacterial action, reducing *S. enterica* and *E. coli* in the ceca of pigs or poultry (Galfi and Neogrady, 1996; Sunkara et al., 2011). Future studies should examine intestinal mucosal histology to determine whether supplementation with *M. elsdenii* induces morphological changes to the gut. Future research also should analyze diarrheal incidence and pathogen presence in the GIT and feces to test whether disease incidence and transmission is affected by supplementation with this DFM.

Conclusion

In summary, supplementation with *M. elsdenii* appears to be effective in altering fermentation in the swine large intestine. Stabilization of fermentation by preventing a decline in microbial diversity and promoting growth and activity of beneficial microorganisms, as well as increased production of butyrate observed in response to supplementation with *M. elsdenii* may improve gut health and absorptive capacity in piglets. This has the potential to decrease their risk of digestive disturbances and improve growth performance. Further research should be conducted to further elucidate the effect of *M. elsdenii* on feed intake in the nursery, piglet growth performance and the long-term effects on performance of *M. elsdenii* administration during the critical peri-weaning period.

Limitations

Feed intake of sows was not monitored on this study. In future studies, it would be valuable to understand the effect of *M. elsdenii* supplementation on sow intake during the lactation period when "starvation sterility" is a risk. During the suckling period, pigs in some litters were supplemented with milk replacer which may have been a confounding factor when evaluating the effect of *M. elsdenii* on growth performance of piglets. There were also large

differences in litter size, although pigs were cross fostered to attempt to normalize litter size. Although measures were taken to prevent direct contact of piglets in the Nn and Yn treatment groups with the probiotic, there is a potential that cross-contamination occurred between treatment groups, although this was unable to be evaluated as PCR was unsuccessful. Piglets were also not blocked by body weight before the administration of the oral gavage of *M. elsdenii*. In future experiments, piglets should be blocked by body weight to account for differences in growth performance caused by initial differences in vitality. Differences in initial body weight make it difficult to derive definitive conclusions regarding the effect of *M. elsdenii* supplementation on growth performance of piglets, as those born at greater body weights generally perform better in each stage of production compared to their lighter weight litter-mates. It would also be beneficial to evaluate whether *M. elsdenii* affects low or high birth weight categories differently. As Feldpausch et al. (2016) suggested, intervention methods may be most effective in improving survivability and growth in these less thrifty pigs compared to their heavier, more vital litter mates. Finally, evaluation of the piglets' intestinal mucosa would be valuable in determining the effect of *M. elsdenii* supplementation on GI health and recovery from weaning.

Table 3.1 Treatment allocation of *Megasphaera elsdenii* oral gavage to sows and their litters

		Megasphaera oral gavage	
Sow	**No. pigs in litter**	**Sow [a]**	**Piglets [b]**
1	13	Y	Y
2	12	N	Y
3	5	N	N
4	12	Y	N
5	11	Y	Y
6	12	N	Y
7	10	Y	N
8	9	N	N
9	11	Y	N
10	11	Y	Y
11	12	N	Y
12	11	N	N
13	11	N	Y
14	12	N	N
15	10	Y	N
16	11	Y	Y
17	10	N	Y
18	11	N	N
19	13	Y	N
20	12	Y	Y
21	14	N	N
22	10	Y	N
23	11	N	Y
24	13	Y	Y
25	15	Y	N
26	14	N	Y
27	11	N	N
28	14	Y	Y

[a] Sow Y received 25 mL *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 1 to 3 d prior to farrowing; Sow N did not receive *M. elsdenii* culture directly

[b] Piglet Y received 5 mL *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 48 h after birth and at weaning; Piglet N did not receive *M. elsdenii* culture directly

Table 3.2 Description of experimental swine diets

	Diet Phase *, †	
Ingredient, % of diet	**Gestation**	**Lactation**
Ground corn	73.25	65.15
Dehulled soybean meal	18.50	24.00
Liquid energy[a]	1.00	2.00
L-lysine hydrochloride 50.7%[b]	0.05	0.45
Magnesium Oxide	0.10	0.10
Potassium chloride[c]	0.10	0.20
Vitamin mineral premix[d]	5.00	5.00
DL-Methionine	0.00	0.10
Protein and energy supplement[e]	2.00	3.00

*Ralco-Advanced Birthright Nutrition Baby Pig Milk Replacer, nonmedicated; used to supplement large litters
†Lean Start® 3 Complete (Hubbard Feeds, Inc., Mankato, MN). Fed *ad libitum* during the nursery period
[a]Vit-e-men, Norfolk, NE
[b] Biolys® Evonik Nutrition and Care GmbH, Essen, Germany
[c] Dyna-K KCL® Mosaic Co., Plymouth, MN
[d] XFE sow base PH, Custom Feeds, Norfolk, NE
[e] EXL Lipex, EXL Milling, Lloyminster SK, Canada

Table 3.3 Effects of *Megasphaera elsdenii* on sow litter size and lactational body weight loss

Item	Sow Y	Sow N	SEM	*P*-value[τ]
Litter size	12.0	12.4	0.99	0.77
Pre-farrowing BW, kg	260.36	267.17	7.500	0.53
Post-farrowing BW, kg	237.16	235.57	10.553	0.92
Lactational weight loss, % BW	-4.10	-6.00	1.517	0.26

Y = Sows received 25 ml *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 1 to 3 d prior to farrowing
N = Sows did not receive *M. elsdenii* culture directly
[τ] Treatment effect

Table 3.4 Effects of *Megasphaera elsdenii* on piglet growth performance, feed intake and mortality

Item						
Sow Treatment	**N**		**Y**			
Piglet Treatment	**n**	**y**	**n**	**y**	**SEM**	***P*-value[τ ‡]**
Nursery						
Birth weight, kg	1.38^{A}	1.79^{C}	1.32^{A}	1.60^{B}	0.070	S, P, I
Weaning weight, kg	6.41^{AB}	6.69^{B}	5.50^{C}	6.11^{A}	0.294	S, P
Nursery ADG, kg						
Starter	0.24^{A}	0.25^{A}	0.22^{B}	0.23^{AB}	0.010	S
Week 1 BW, kg	6.85^{A}	7.33^{B}	5.96^{C}	6.75^{A}	0.292	S, P
ADFI, kg	0.12^{A}	0.14^{AB}	0.12^{A}	0.15^{B}	0.011	P
ADG, kg	0.05^{A}	0.09^{B}	0.06^{A}	0.09^{B}	0.009	P
Grower						
Week 2 BW, kg	8.85^{A}	9.54^{C}	8.18^{B}	8.82^{A}	0.348	S, P
Week 3 BW, kg	11.83^{AC}	12.54^{C}	10.85^{B}	11.64^{A}	0.430	S, P
Week 4 BW, kg	16.02^{AC}	16.69^{C}	14.23^{B}	15.33^{A}	0.461	S, P
ADFI, kg	0.30	0.34	0.26	0.29	0.040	--
ADG, kg	0.44^{AC}	0.45^{A}	0.39^{B}	0.41^{BC}	0.017	S
Total post-wean						
ADFI, kg	0.25	0.29	0.22	0.26	0.030	--
ADG, kg	0.34^{A}	0.36^{A}	0.31^{B}	0.33^{AB}	0.013	S, P ‡
Total						
Overall ADG kg	0.30^{AC}	0.31^{A}	0.28^{BC}	0.29^{C}	0.010	S, P ‡
Mortality, %	8.2	5.3	9.0	10.6	4.88	--

N = Sows did not receive *M. elsdenii* culture directly
Y = Sows received 25 mL *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 1 to 3 d prior to farrowing
n = Piglets did not receive *M. elsdenii* culture directly
y = Piglets received 5 mL *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 48 h after birth and at weaning
τ S = effect of treating sows with *M. elsdenii;* P = effect of treating litters with *M. elsdenii;* I = interaction effect of sow treatment by piglet treatment*; P* ≤ 0.05
‡ S ‡ = effect of treating sows with *M. elsdenii;* P ‡ = effect of treating litters with *M. elsdenii; P* ≤ 0.10
A, B Values within a row with different letters are different at *P* ≤ 0.05

Table 3.5 Effects of *Megasphaera elsdenii* on sow fecal pH and VFA

	Day 0		Day 7		Day 14		Day 21			
Item*	**Y**	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**	**SEM**	***P*-value ᵀ ‡**
pH	6.74	6.83	6.69	6.73	6.73	6.73	6.72	6.68	0.073	--
Total VFA	73.00	64.60	112.67[1]	90.10[2]	146.29	147.55	150.22	150.26	8.496	D
Acetate	41.11	38.38	61.73	53.01	78.87	82.98	83.02	83.13	4.728	D
Propionate	16.17	13.05	25.36[A]	18.50[B]	35.06	32.47	36.05	34.63	2.286	S, D
Butyrate	6.91	5.74	13.19[A]	9.29[B]	16.54	16.07	16.51	16.69	1.547	D
Isobutyrate	2.15	1.88	3.04[1]	2.03[2]	4.10	4.48	3.06[1]	4.00[2]	0.390	D, I ‡
Valerate	2.00	1.71	2.93	2.33	4.16	3.71	4.03	3.93	0.294	S ‡, D
Isovalerate	3.06	2.75	5.46[A]	4.16[B]	6.99	7.03	6.94	7.35	0.469	D
Caproate	0.60[A]	0.25[B]	0.41	0.28	0.29	0.32	0.31	0.18	0.103	S
Isocaproate	0.461	0.289	0.193	0.169	0.121	0.177	0.136	0.100	0.0889	D
Heptanoate	0.539	0.427	0.357	0.215	0.150	0.177	0.157	0.123	0.1440	D

*VFA concentrations reported in mM
Day = days post-farrowing
Y = Sows received 25 mL *M. elsdenii* culture (2 x 10^9 CFU/mL) as an oral gavage 1 to 3 d prior to farrowing
N = Sows did not receive *M. elsdenii* culture directly
[A, B] Values within a day with different letters are different at $P \leq 0.05$
[1, 2] Values within a day with different numbers are different at $P \leq 0.10$
ᵀ S = effect of treating sows with *M. elsdenii;* D = effect of day; I = Interaction effect of sow treatment and day, $P \leq 0.05$
‡ S ‡ = effect of treating sows with *M. elsdenii;* I ‡ = Interaction effect of sow treatment and day, $P \leq 0.10$

Table 3.6 Effects of *Megasphaera elsdenii* on piglet fecal pH and VFA profile

Item*	Day 0				Day 7				Day 14				Day 21				Day 28					
Sow trt	Sow N		Sow Y		Sow N		Sow Y		Sow N		Sow Y		Sow N		Sow Y		Sow N		Sow Y			
Piglet trt	n	y	n	y	n	y	n	y	n	y	n	y	n	y	n	y	n	y	n	y	SEM	*P*-value [τ]
pH	6.89^{A}	7.20^{B,}	7.17 ^{B}	7.05 ^{AB}	6.67^{A}	6.65^{A}	6.62^{A}	6.62^{A}	6.55^{A}	6.68^{A}	6.56^{A}	6.57^{A}	6.33^{A}	6.45^{AB}	6.63^{B}	6.54^{AB}	6.32 ^{A}	6.47^{A}	6.36^{A}	6.26 ^{A}	0.104	T
Total VFA	52.24^{A}	43.70^{A}	47.65^{A}	45.14^{A}	56.94^{A}	72.52^{B}	69.79^{AB}	81.06^{B}	66.66^{A}	96.00^{BC}	90.41^{B}	103.90^{C}	77.79^{A}	79.49^{A}	86.75^{A}	103.95^{B}	61.28^{A}	95.82^{B}	59.08^{A}	95.15^{B}	5.255	SD, PD
Acetate	30.42^{A}	26.93^{A}	29.27^{A}	27.53^{A}	33.46^{A}	41.35^{BC}	39.05^{AB}	46.27^{C}	44.88^{A}	61.33^{BC}	59.28^{B}	66.03^{C}	46.63^{A}	52.82^{AB}	58.67^{B}	69.94^{C}	41.91^{A}	60.06^{B}	40.81^{A}	57.93^{B}	2.935	SD, PD,
Propionate	8.19^{A}	7.84^{A}	7.77^{A}	8.24^{A}	13.73^{A}	17.18^{B}	17.54^{B}	18.09^{B}	12.50^{A}	19.65^{BC}	17.69^{B}	21.40^{C}	17.46^{A}	15.14^{A}	15.69^{A}	18.16^{A}	11.27^{A}	19.38^{B}	10.58^{A}	19.77^{B}	1.372	PD
Isobutyrate	3.33^{A}	3.09^{A}	3.18^{A}	2.04^{B}	1.17^{A}	1.73^{B}	1.70^{AB}	2.01^{B}	0.82^{A}	1.66^{B}	1.40^{B}	1.47^{B}	1.15^{AB}	0.99^{A}	1.19^{AB}	1.56^{B}	0.77^{A}	1.73^{B}	0.70^{A}	1.78^{B}	0.219	I
Butyrate	4.20^{A}	2.15^{A}	2.74^{A}	2.94^{A}	5.37^{A}	7.48^{BC}	6.64^{AB}	8.72^{C}	6.07^{A}	8.92^{B}	8.40^{B}	10.79^{C}	9.63^{AB}	7.93^{A}	8.20^{A}	10.34^{B}	5.34^{A}	10.27^{B}	5.10^{A}	10.77^{B}	0.829	PD, T^{ε}
Isovalerate	3.74^{A}	2.48^{B}	2.91^{B}	2.98^{B}	1.92^{A}	2.81^{B}	2.81^{B}	3.19^{B}	1.23^{A}	2.44^{B}	2.07^{B}	2.38^{B}	1.66^{AB}	1.43^{A}	1.61^{AB}	2.14^{B}	1.12^{A}	2.50^{B}	1.08^{A}	2.62^{B}	0.300	I
Valerate	1.33^{A}	0.52^{B}	0.99^{AB}	0.68^{B}	1.19^{A}	1.87^{B}	1.89^{B}	2.50^{C}	1.07^{A}	1.90^{B}	1.49^{AB}	1.74^{B}	1.21^{AB}	1.04^{A}	1.23^{AB}	1.65^{B}	0.81^{A}	1.84^{B}	0.82^{A}	2.21^{B}	0.241	PD, S
Caproate	0.518^{A}	0.123^{B}	0.167^{B}	0.000^{B}	0.066^{AB}	0.024^{A}	0.157^{AB}	0.248^{B}	0.071^{A}	0.106^{A}	0.083^{A}	0.080^{A}	0.021^{A}	0.143^{A}	0.165^{A}	0.148^{A}	0.027^{A}	0.018^{A}	0.000^{A}	0.059^{A}	0.0815	SD, PD

*All VFA concentrations reported in mM
Day = days post-weaning
Y = Sows received 25 mL *M. elsdenii* culture (2 x 10^{9} CFU/mL) as an oral gavage 1 to 3 d prior to farrowing
N = Sows did not receive *M. elsdenii* culture directly
y = Piglets received 5 mL *M. elsdenii* culture (2 x 10^{9} CFU/mL) as an oral gavage 48 h after farrowing and at weaning
n = Piglets did not receive *M. elsdenii* culture directly
$^{A, B}$ Values within a day with different letters are different at $P \leq 0.05$
S = effect of treating sows with *M. elsdenii;* SD = interaction effect of sow treatment and day; PD = interaction effect of piglet treatment and day; T = interaction effect of sow treatment and piglet treatment; I = interaction effect of sow treatment, piglet treatment and day, $P \leq 0.05$
^{ε} I = interaction tendency of sow treatment, piglet treatment and day; T = interaction tendency of sow treatment and piglet treatment, $P \leq 0.10$

Chapter 4 – Evaluation of the effects of *Megasphaera elsdenii* strain NCIMB 41125 on fermentation within the equine cecum

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Abstract

This study was conducted to determine effects of supplementation with lactate-utilizing *Megasphaera elsdenii* strain NCIMB 41125 (MSBiotec, Wamego, Kansas) on equine cecal pH and volatile fatty acid (VFA) profile when dietary roughage to concentrate ratio was decreased. Eight cannulated American Quarter horses (Beard et al., 2011) were used in an incomplete 3 x 3 Latin square design, with 3, 7-d treatment periods separated by 28-d washouts. Treatment 1 was a negative control (NC), which had no direct contact with the probiotic culture. Treatment 2 consisted of 50-mL oral drench (OD) containing 2.12 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125, administered at the onset of each period. Treatment 3 horses received approximately 0.80 g of lyophilized powder (LP) containing 8.58 x 10^9 CFU/g of *M. elsdenii* strain NCIMB 41125, administered daily within 2 commercially produced equine pill pockets (Uncle Jimmy's Squeezy Buns Pliable Pill Pocket Horse Treats, Uncle Jimmy's, New Castle, PA). While in washout periods, horses were fed only Smooth Bromegrass hay. During each treatment period, horses were started at a 90:10 roughage to concentrate diet and gradually stepped up over 5 d to a 50:50 roughage to concentrate diet by d 5. Cecal samples were collected every 4 h for pH and VFA analyses. Horses supplemented with *M. elsdenii* tended to exhibit greater cecal pH than the NC animals as the roughage to concentrate ratio decreased ($P < 0.10$). The greatest pH differences were noted at the maximum level of concentrate inclusion, on d 5 and 7 in control compared to OD and LP horses, respectively ($P < 0.10$). Cecal acetate to propionate ratio (A:P), valerate and caproate concentrations were greater in LP than NC on d 7 ($P < 0.05$). Cecal isobutyrate and isovalerate concentrations decreased in LP horses on d 7, correlating with increased A:P ($P < 0.05$). *M. elsdenii* effectively altered the cecal environment. Increased production of the 5-carbon fatty acid, valerate, likely contributed to the increased

acetate to propionate ratio, as it is a growth factor for fibrolytic, acetate-producing bacteria. Elevated A:P may also be attributed to greater cecal pH in LP and OG horses than the control, creating a favorable environment for fermentation of fiber. Further studies should be conducted utilizing more severe or prolonged carbohydrate challenge to ascertain the ability of *M. elsdenii* to mitigate negative consequences of increasing concentrate inclusion in the diet.

Introduction

Horses are hindgut fermenters, possessing specialized microbial populations in their cecum and colon that are well adapted to continuous grazing of fibrous forage. In a natural setting, horses will graze up to 16 h per day, resulting in continuous flow of fibrous substrate through their gastrointestinal tracts (GIT). However, domestication has disrupted this natural grazing pattern, partly due to the addition of concentrates, containing large amounts of hydrolyzable carbohydrates, such as starch (Daly et al., 2012). If starch is fed in excess of 0.2 to 0.4% body weight per meal, depending on the source, it exceeds the threshold for enzymatic digestion in the small intestine (Potter et al., 1992; Kienzle et al., 1994). Starch that escapes the small intestine will undergo rapid fermentation in the hindgut. Accelerated fermentation causes a drastic reduction in pH, triggering shifts in microbial populations of the cecum and colon resulting in dysbiosis (van den Berg et al., 2013). Starch is the preferred substrate for amylolytic, lactate-producing bacteria (LAB). Proliferation of these species occurs at the expense of acetate and butyrate producing fibrolytic bacteria (Daly et al., 2012). This microbial imbalance is associated with issues such as decreased fiber degradation or potentially fatal gastrointestinal-related disorders, such as colic and laminitis (Rowe et al., 1994; Milinovich et al., 2007; Durham, 2009; Milinovich et al., 2010; Van den Berg et al., 2013). Millinovich et al. (2007, 2010) believe that laminitis can be linked to the proliferation of LAB, *Streptococcus bovis, S. equinus,* and *Lactobacillus* spp. Over-production of lactate by LAB is also accompanied by CO_2 production which leads to gastrointestinal distention and potentially colic. Digesta also tends to contain less moisture in conditions where accumulation of lactate is occurring which may predispose the horse to impaction colic (Shirazi-Beechey, 2008).

Probiotics have been tested in horses as a means of promoting microbial balance and mitigating adverse effects of feeding high grain diets with variable results. The most popular probiotic for use in the equine is *Saccharomyces cerevisiae* (SC), or yeast (Schoster et al., 2014). Yeast, if provided daily, have been shown to increase lactate-utilizing bacterial (LUB) populations in the hindgut and prevent the dramatic decline of pH associated with diets containing high inclusion levels of starch-rich concentrate. This is also associated with increased fiber degradation in supplemented animals (Medina et al., 2002; Jouany et al., 2009); however, these results have been contradicted in other studies. Taran et al. (2016) studied effects of yeast supplementation of protein and fiber digestibility and fecal parameters, and found that the probiotic did not affect fecal pH, microbial profile or nutrient digestibility in horses fed either a high-concentrate or high-forage diet. Lattimer et al. (2007) and Mackenthun et al. (2013), also found that SC did not alter fermentation or fiber digestion in supplemented horses.

In order to be effective, yeast must be provided daily as they do not colonize the equine hindgut (Schoster et al., 2014); thus supplementation with a microorganism possessing the ability to colonize the hindgut may provide more consistent results. A previous study in our laboratory confirmed the delivery and survival of *M. elsdenii* in the equine hindgut when the bacterial culture was administered orally (Dove et al., unpublished). This lactate-utilizing bacterium has been used effectively in ruminant animals to stabilize pH and prevent excessive accumulation of lactate upon introduction to a high-grain diet (Henning et al., 2009; 2010). Others have reported *M. elsdenii* decreases the incidence of diarrhea and bloat in feedlot cattle and morbidity in dairy cattle, following rapid transition to high-concentrate diets (Leeuw et al., 2009; Hagg et al., 2010).

Previous *in vitro* work conducted in our laboratory examined the effect of adding *M. elsdenii* to equine cecal cultures containing either inulin or corn starch to induce acidosis. Lactate

accumulation decreased with the addition of *M. elsdenii* in equine cecal cultures containing corn starch. In cultures containing both inulin and corn starch, *M. elsdenii* increased fermentative activity and dry matter disappearance (Leventhal et al., 2011). The effects of *M. elsdenii* supplementation *in vivo* have yet to be established in the horse. Therefore, this study was designed to evaluate the response of equine cecal parameters to supplementation with lactolytic *M. elsdenii*, and its potential for use in mitigating negative repercussions of feeding diets containing rapidly fermentable concentrate.

Materials and methods

All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Facilities

Horses were housed in individual stalls (3.05 x 3.66 m) within a single barn and bedded with pine shavings. Horses were randomly assigned to different stalls for each treatment period to account for any possible variation in ventilation or temperature based on location in the barn. Horses were walked daily for exercise during treatment periods.

Animals, treatments and diets

Eight mature American Quarter horses, 4 mares and 4 geldings between the ages of 7 and 11 years (average BW = 540 kg; SEM = 75 kg), previously fitted with cecal cannulae (Beard et al., 2011), were used in a 3 x 3 (treatment x horse) incomplete Latin square replicated over 3 treatment periods. Each 7-d treatment period was separated by a 28-d washout period. Horses were randomly assigned to treatments within period. The 3 treatments consisted of a negative control (no *M. elsdenii;* NC), 50 mL of fresh culture containing 2.12 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 (MSBiotec, Wamego, Kansas) administered via oral drench (OD),

and approximately 0.80 g of a lyophilized powder culture containing 8.58 x 10^9 CFU/g of *M. elsdenii* strain NCIMB 41125 contained within 2 commercially produced equine pill pockets (Uncle Jimmy's Squeezy Buns Pliable Pill Pocket Horse Treats, Uncle Jimmy's, New Castle, PA) (LP; Table 4.1).

Administration of treatments

Horses in the OD treatment group were dosed just prior to feeding on d 1 of each treatment period with 50 mL of fresh culture containing 2.12 x 10^9 CFU/mL of *M. elsdenii* strain NCIMB 41125 using a manually operated dosing device (60 mL Variable Automatic Drencher MKIII, NJ Phillips, NSW, Australia). Prior to administration of the probiotic culture, a 5-L bag of fresh culture was shaken vigorously to homogenize contents. A manually operated dosing device was attached to the bag using Tygon tubing and the reservoir was filled. Approximately 100 to 200 mL of the culture was discarded into a waste container to ensure that both the tubing and the device would be devoid of oxygen. The oral dosing syringe was then placed directly into the horse's mouth and the contents expelled. Horses' mouths were held closed until swallowing occurred to ensure product could not be ejected from the mouth.

M. elsdenii strain NCIMB 41125 was freeze-dried in advance of the study and packaged in anaerobic vacuum sealed packets. Each packet contained approximately 0.39 g of the freeze-dried bacterium containing 8.58 x 10^9 CFU/g of *M. elsdenii*. Horses in the LP treatment group were offered 2 commercially produced equine pill pockets (Uncle Jimmy's Squeezy Buns Pliable Pill Pocket Horse Treats, Uncle Jimmy's, New Castle, PA) containing 1 packet of the lyophilized product just prior to the morning feeding each day during the treatment period. Packets containing the lyophilized powder were kept frozen at -18^o C until just prior to administration of treatments each day during the treatment period. The pill pocket was broken open and contents

of 1 packet of powdered culture pressed directly into the pill pocket treat and offered to LP horses. If horses refused the treat, the freeze-dried product was manually placed on the back of the tongue and the horse's mouth held closed until the bolus was swallowed. The contents of one packet containing the lyophilized powdered culture were plated each day to ensure consistent bacterial viability through each treatment period (Table 4.2). Remaining horses had no direct exposure to the probiotic during the treatment period in which they served as controls.

Feeding and watering

During treatment periods, horses were fed hay and concentrate split equally between the 2 daily feedings. Each horse was fed 1% of its body weight (BW) as fed (AF) in brome hay per day (Table 4.3). Horses were stepped up to 1% BW AF in textured concentrate (analysis in Table 4.3; composition in Table 4.4) at a rate of 0.2% BW AF per day on d 1 through d 5, then maintained at 1% BW AF for d 5 through d 7. Refusals were infrequent, but each refusal was weighed and recorded. Stalls were equipped with automatic waterers to accommodate *ad libitum* consumption. Waterers were cleaned and checked for proper function multiple times per day.

During each 28-d washout period, horses were housed together in a dry lot and maintained on an *ad libitum* brome hay diet. Horses were weighed at the termination of each washout period to ensure accurate calculation of the amount of feed offered during treatment periods.

Sample collection

Cecal samples were collected via cecal cannulae every 4 h during each 7-d treatment period. Horses were fed at 1000 h and 2200 h each day and samples were collected at 4, 8 and 12 h post-feeding, with the 12 h sample obtained just prior to the next feeding. On d 0 of each

treatment period, cecal samples were collected prior to dosing or feeding to establish baseline values of pH, VFA and *M. elsdenii* populations in the hindgut.

Samples were collected by removing the cannulae caps and catching cecal contents as they flowed out of the cannulae. Cecal fluid was strained through 4 layers of cheesecloth, and then placed into a 100-mL specimen cup. If a sufficient sample was not collected via gravity flow, a handheld pump was used to extract cecal contents. At 1000 h on d 0, 1, 3 and 7, at least 20 mL of additional cecal digesta were collected for PCR analysis. During the first treatment period, unfiltered samples were collected in 20-mL HDPE scintillation vials (Fischer Sci.; 03-337-23B). These unfiltered samples posed a challenge in separating samples for DNA extraction, so for the remaining treatment periods, at least 15 mL of strained cecal fluid was collected in 50-mL Falcon conical centrifuge tubes (Corning Inc. 352070; Corning, NY) and immediately frozen at -80^{o}C to await PCR analysis. Technicians changed gloves between each horse.

Sample analyses

Cecal pH was measured immediately after collection using a portable pH meter (Thermo Scientific Orion 3 Star Portable pH Meter, Waltham, MA; Accumet probe). After pH was recorded, the sample was transferred in 1 mL aliquots into 2 microcentrifuge tubes and mixed with 0.25 mL of 25% meta-phosphoric acid for deproteinization. Samples were frozen at -18^{o}C for at least 24 h prior to VFA analysis.

Acidified and frozen cecal samples were thawed and homogenized using a vortex mixer (Scientific Industries Vortex-Genie 2 vortex mixer, Houston, TX). They were then centrifuged at 24 x g for 18 min. The aqueous supernatant was then transferred into gas chromatography vials. Volatile fatty acids were measured using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a DB-WAX capillary column (10 m x 0.10 mm x

0.1 mm film thickness; Agilent and J&W columns, Santa Clara, CA) and flame ionization detector. Hydrogen was used as a carrier gas at a flow rate of 46 cm/sec, with a 1-μL split injection and a split flow 50:1. Initial oven temperature was 70°C and temperature was increased by 15°C/min to 130°C, then increased at 60°C/min to 220°C and held for 2 min. Inlet and detector temperatures were 260°C and 300°C respectively. Volatile fatty acids were quantified by comparing 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.

Statistical analyses

Data were analyzed using the Glimmix procedure of SAS Version 9.4. The model included the fixed effect of treatment and random effects of horse, period, and treatment by period interaction. Horse served as the experimental unit. The treatment by hour within day effect was not significant for any parameter and was therefore excluded from the model. Significance was declared at $P \leq 0.05$, and 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

Freeze-drying efficacy

The freeze-drying procedure effectively maintained viability of *M. elsdenii* for delivery to horses in powdered form. Rehydrated samples of the lyophilized powdered culture, plated during each treatment period, revealed mean counts of 8.58×10^9 CFU/g per dose.

PCR

Due to unforeseen difficulties with the DNA extraction step of the analysis, PCR was unsuccessful. Therefore, differences in populations of *M. elsdenii* in the equine hindgut between treatments was unable to be evaluated.

Cecal pH

No treatment by hour or treatment by hour within day interactions were detected for cecal pH, therefore treatment by day interaction effect was considered. As the dietary roughage to concentrate ratio decreased, mean cecal pH declined in all horses (Fig 4.1). However, cecal pH tended to be greater in horses treated with *M. elsdenii* compared to controls as the inclusion of grain in the diet increased ($P < 0.10$). Cecal pH of OD horses was elevated above NC on d 5, the first day in which the full allotment of grain was fed (7.00 and 7.19 respectively; $P < 0.10$). On d 7, LP horses tended to have greater cecal pH (7.19) than NC (6.99; $P < 0.10$).

Cecal VFA profile

No treatment by hour or treatment by hour within day interactions were detected for cecal VFA concentrations, therefore treatment by day interaction effects were considered. Supplementation with *M.* had no effect on total acetate or propionate concentrations in the cecum ($P > 0.60$; Table 4.5). A day effect was detected in the cecal acetate, propionate, acetate to propionate ratio (A:P), butyrate and total VFA concentrations. The effect of day corresponds with increasing grain inclusion in the diet. Cecal acetate decreased from d 0 to d 6 or 7 in all treatments. In NC horses, mean acetate concentration was 47.08 mM on d 0 and declined to 38.42 mM by d 7 ($P < 0.05$). Acetate decreased from 43.29 to 37.48 mM on d 7 ($P = 0.10$) in OD animals. Finally, in LP horses, acetate concentrations fell from a baseline of 46.86 to 36.71 mM on d 6 ($P < 0.05$), but increased on d 7 to 38.34 mM, analogous to d 5 concentrations ($P > 0.20$).

Propionate concentrations initially increased from d 0 to d 5 in OD and NC treatment groups and then decreased to levels analogous with d 0 on d 6 and 7. In the LP treatment group, cecal propionate concentrations remained constant from d 0 to d 5, then decreased from d 5 to d 6 and 7. Cecal acetate to propionate ratio (A:P) decreased from d 0 to d 5 for all treatments ($P < 0.05$). In NC horses A:P remained constant from d 5 to d 7 ($P > 0.50$).The A:P in OD and LP horses remained constant from d 5 to 6, then increased on d 7 ($P < 0.05$). On d 7, cecal A:P was greater in LP horses (2.83) than in NC (2.41) or OD horses (2.60; $P < 0.05$). Butyrate also decreased from d 0 to d 7 in NC horses ($P < 0.05$). However, butyrate levels remained constant from d 0 to d 7 in OD and LP horses ($P > 0.10$). Caproate concentrations in the cecum were unaffected by treatment or day in this study ($P > 0.10$). Total VFA concentrations were similar on d 0 and d 7 in all treatment groups ($P > 0.05$). Total cecal VFA decreased from d 5 to d 7 in NC and OD treatment groups ($P < 0.02$). In LP horses, concentrations of total VFA tended to decrease from d 5 to d 7 ($P = 0.06$).

A treatment by day interaction was identified for valerate, isobutyrate and isovalerate. Cecal valerate concentration of NC horses remained constant from d 0 to d 6, then dropped from d 6 (0.114 mM) to d 7 (0.033 mM; $P < 0.01$). No differences were observed in cecal valerate concentrations between days in OD and LP animals ($P > 0.15$). Valerate concentration was greater on d 7 in the LP treatment group (0.123 mM) than NC (0.033 mM; $P < 0.05$), though it was comparable to that of the OD treatment group (0.059 mM; $P > 0.10$). Valerate concentration was less in OD horses on d 5 than the NC animals ($P < 0.01$), however, it was similar to that of LP horses ($P > 0.10$). Cecal isobutyrate and isovalerate concentrations were greater on d 0 in LP horses than either NC or OG ($P < 0.05$), which were similar to each other ($P > 0.90$).

Concentrations of these branched chain fatty acids also decreased from d 0 and 5 to d 7 in LP horses ($P < 0.05$).

Heptanoate and isocaproate concentrations were negligible, and no treatment nor interaction effects were detected; thus, these VFA results were excluded from Table 4.4. In the second treatment period two VFA samples from horse 6, 1 from d 6 and 1 from d 7, 1 sample from horse 7 on d 7 and 1 sample from horse 2 on d 6 were contaminated with water after thawing and therefore, VFA data from those 4 samples were not included in the analysis.

Discussion

Increasing the level of grain inclusion in a horse's diet can have deleterious effects on the hindgut, including cecal acidosis. Under normal conditions, LUB will take about 7 to 12 d to increase population to a sufficient density to ferment excess lactate produced in response to marked increase in dietary grain inclusion. This fermentation of lactate produced by saccharolytic bacteria minimizes lactate accumulation and pH fluctuation in the cecum of the horse (Goodson et al., 1988; Klieve et al., 2003). If the transition period to a high concentrate diet is not sufficient to allow establishment of LUB populations, accumulation of lactate will occur. Lactate accumulation related to increasing hydrolyzable carbohydrate concentrations in the equine diet result in a decline in cecal pH. This is associated with digestive disorders such as colic or laminitis, as well as undesired behavioral changes. Adverse behaviors noted as a result of cecal acidity include "wind sucking", "cribbing" or consuming bedding (Rowe et al., 1995; Milinovich et al., 2007). Starch content was not evaluated in the experimental concentrate, however given the ingredient composition of the concentrate, we calculate it to contain approximately 50% starch on a dry matter basis. Therefore, horses received approximately 0.22% of their body weight in starch per feeding which falls within the range established by

Potter et al. (1992) and Kienzle et al. (1994) exceeding the threshold for prececal digestion of starch.

Cecal pH decreased from d 0 to d 5 across all treatment groups as expected with increasing dietary grain inclusion (Goodson et al., 1988). While there were generally no differences detected in cecal pH between treatments, on d 5 and d 7 when the maximum amount of grain was consumed, OD and LP horses, respectively, tended to have greater cecal pH than the control. Greater cecal pH indicates that supplementation with *M. elsdenii* prior to addition of grain to the diet may help attenuate the reduction in cecal pH caused by increased dietary grain inclusion. Future studies with greater numbers of horses would add statistical power to these data and likely make it possible to detect greater cecal pH differences among treatments. The hypothesis that supplementation of horses with *M. elsdenii* would minimize pH decline in the cecum is in agreement with Leventhal et al. (2011) who reported a similar ability of *M. elsdenii* to attenuate pH reduction *in vitro* using a cecal fluid inoculum with corn starch or fructans as the substrate. Published studies using *M. elsdenii* in the horse are almost non-existent; therefore, a comparative assessment with ruminant data is required. Klieve et al. (2003) and Henning et al. (2009; 2010), reported that ruminal pH declined to a lesser degree in cattle or sheep supplemented with *M. elsdenii* compared to un-supplemented animals when abruptly transitioned to high-grain diets. Long et al. (2014) reported that *M. elsdenii* mitigates lactate accumulation and pH decline *in vitro* when acute ruminal acidosis was simulated.

Although pH tended to be greater in treated horses compared to controls on d 5 and 7, differences in pH were not reflected in cecal VFA concentrations. *Megasphaera elsdenii* is a known lactate-utilizing microorganism and has the ability to ferment either glucose or lactate to produce VFA and organic acids (Lewis and Elsden, 1955; Counotte et al., 1981; Hino et al.,

1994; Kung and Hession, 1995; Prabhu et al., 2012). Thus, *M. elsdenii* can control pH decline through several modes of action. The first is through conversion of lactate into predominately propionate or valerate, with the production of some butyrate and acetate (Marounek et al., 1989; Kung and Hession, 1995). The second is through fermentation of glucose. Glucose fermentation serves the dual purpose of generating VFA for energy and competing with saccharolytic LAB for substrate, thus reducing the production of lactate. Butyrate and acetate are the primary glucose fermentation products of *M. elsdenii*, with some strains also producing small amounts of caproate (Marounek et al., 1989; Hino and Kuroda, 1993). However, no increase in cecal acetate, propionate, butyrate or total VFA concentrations were observed in treated horses in this study. Great inter-horse variability and small sample size resulted in high standard errors, making treatment differences difficult to detect. As horses were stalled adjacent to each other during treatment periods, it is also possible that cross-contamination between treatments may have occurred, although control horses had no direct contact with the probiotic culture.

Cecal acetate concentrations are expected to decrease as dietary grain increases. Propionate- and lactate-producing saccharolytic microorganisms outcompete acetate-producing, fibrolytic species for available substrate under these conditions (Daly et al., 2012). Our results reflect this predictable decrease. Delivery of *M. elsdenii* to the hindgut should result in competition for substrate with LAB. The ability of *M. elsdenii* to compete for substrate with LAB such as *S. bovis and S. equinus* reduces the risk of disorders associated with proliferation of these bacterial species and may aid in minimizing the decline of fibrolytic bacterial populations that often accompanies proliferation of LAB, thus promoting microbial balance (Allison et al., 1958; Nagaraja and Titgemeyer, 2007; Daly et al., 2012; Zhang et al., 2013). Large standard errors in cecal propionate and acetate concentrations likely resulted in the lack of differences

detected between treatments for these parameters. Although no treatment by day interaction was detectable in cecal A:P, LP horses had numerically greater A:P on d 7 than either OD or NC, coinciding with pH differences detected between LP and NC horses. Increased cecal A:P, on d 7 in LP horses, may be due to greater cecal pH associated with supplementation of the bacterium on this day, over the controls. Greater cecal pH correlates with increased acetate production due to the creation of a more amenable environment for the establishment of fibrolytic bacteria (Hoover, 1986; Yang et al., 2002). Acetate production has been reported to be maximal at a pH of 7.4 (Satter and Esdale, 1968). Cecal pH in this trial was nearer to 7.4 in OD and LP animals than NC for d 5 and 7 respectively.

Propionate concentrations are expected to increase as grain increases, as it is a major fermentation product of LAB, which are the primary fermenters of the hydrolyzable carbohydrates found in grain (Mackie and Gilchrist, 1979). *Megasphaera elsdenii* is known to produce propionate through the fermentation of lactate via the acrylate pathway and glucose under conditions of minimal lactate availability (Hino et al., 1994; Prabhu et al., 2012). It was expected that propionate concentrations would increase in response to *M. elsdenii* supplementation. The results of this study contradict the findings of Klieve et al. (2003), who reported increased ruminal propionate concentrations in response to *M. elsdenii* while undergoing a rapid transition to a high grain diet.

Butyrate concentrations were unaffected by treatment. However, cecal concentrations of this 4-carbon fatty acid decreased numerically from d 0 to d 7 in NC horses, while remaining constant in OD and LP horses. This may indicate *M. elsdenii* mitigated the decline in butyrate production observed in control animals as dietary grain increased. Maximal fermentation of lactate to form butyrate occurs at a pH of about 6.2 in *in vitro* models of lactate fermentation

(Satter and Esdale, 1968). Average cecal pH in this study never declined to this level. This may account for the absence of expected butyrate increases in the current study, while Leventhal et al. (2011) noted that *M. elsdenii* led to increased butyrate production in equine cecal cultures simulating cecal acidosis where pH fell as low as 4.5. Lack of butyrate utilization *in vitro* also results in greater butyrate concentrations than would be detected *in vivo.*

Greater valerate in LP horses compared to OD or NC horses on d 7 may be due to fermentation of lactate, glucose or amino acids by the bacterium (Lewis and Elsden, 1955; Marounek et al., 1989; Klieve et al., 2003; Shetty et al., 2013). Weimer and Moen (2013) reported metabolism of propionate by *M. elsdenii* to produce valerate. Conversion of propionate to valerate may be another factor contributing to the increased A:P ratio observed on d 7 in LP animals over the other treatment groups. Branched-chain fatty acids, such as isobutyrate and isovalerate, are important growth factors for acetate-producing, fibrolytic bacteria, and are produced by *M. elsdenii* through deamination of branched-chain amino acids (Forsberg, 1978; Marounek et al., 1989; Kung Jr. and Hession, 1995; Zhang et al., 2013). In the current study, isobutyrate and isovalerate, decreased from d 0 to d 7 in LP horses. A decrease in isobutyrate and isovalerate concurrent with increased valerate concentrations was also observed by Zebeli et al. (2012), when *M. elsdenii* was administered to lactating dairy cows. Conversely, Miura et al. (1980) and Allison (1978) reported net production of isovalerate and isobutyrate in *M. elsdenii* culture. It is possible that the washout of 28 days between treatment periods was not sufficient to prevent bacterial carry-over from the previous treatment period. Bacterial carry-over from the previous treatment period could account for the presence of these branched-chain fatty acids on d 0, as well as the lack of treatment differences detected in other VFA concentrations. The decrease in cecal isobutyrate and isovalerate concentrations in LP horses coincides with

increased A:P observed on d 7 for this treatment. Availability of growth factors in conjunction with stabilization of pH, creates an optimal environment for the growth and activity of fiber fermenting microorganisms. The observed decrease in branched chain fatty acids may be due to net utilization by fibrolytic bacteria. This, together with increased A:P, is indicative of improved fiber fermentation, although fiber digestion was not evaluated in this study.

Negligible cecal caproate concentrations were detected in NC and OD horses. However, caproate increased from d 5 to d 7 in LP horses. *Megasphaera elsdenii* is known to produce caproate from the fermentation of glucose and, to a lesser extent, lactate (Miura et al., 1980; Marounek et al., 1989a; Shetty et al., 2013). Antibacterial properties of this 6-carbon fatty acid have been established in poultry studies where caproate has been shown to reduce colonization of *Salmonella* and *E. coli* in the gastrointestinal tract. Thus, elevated production of this VFA may decrease susceptibility to disease and reduce fecal shedding of pathogens (Immerseel et al., 2004). However, Immerseel et al. (2004) used caproate concentrations between 5 and 15 mM to induce antibacterial response, while caproate concentrations in this study remained below 1 mM, it is unlikely that caproate production increased to a sufficient level to affect pathogen colonization in the equine cecum.

Daily application of lyophilized *M. elsdenii* appeared to be more effective than a single dose, administered via oral gavage, in altering cecal parameters, as differences were noted in valerate and branched-chain fatty acid concentrations in LP horses, as well as the numerical differences detected in A:P. This is unsurprising, as daily administration of probiotics generally improves efficacy (Chaucheyras-Durand and Durand, 2009; Collins et al., 2009; Schoster et al., 2014). Previous studies in our laboratory have established the ability of *M. elsdenii* to survive transport through the stomach and small intestine of the equine and reach the cecum in sufficient

numbers to colonize when administered orally (Dove et al., 2014, unpublished data). Although it is likely that *M. elsdenii* reached the cecum when administered as an oral drench, it is probable that daily administration of this direct fed microbial resulted in greater populations of the *M. elsdenii* in the hindgut, compared to animals dosed a single time. This likely explains the fermentation differences observed between the two treatments.

Conclusion

In summary, *M. elsdenii* administered prior to transition to a high grain diet may help to maintain an optimal cecal environment for microbial fermentation in horses; thus, alleviating some of the negative consequences of increased concentrates in the diet. Results of this study suggest that *M. elsdenii* may also be useful in increasing fiber digestion. Future researchers may wish to also examine changes in fiber digestion in the presence of *M. elsdenii*, although Miller (2013) reported decreased NDF digestibility in beef cattle supplemented with *M. elsdenii*. However, Miller's findings were compounded by differing transition periods between control and dosed cattle. Additional studies are warranted to examine the effect of *M. elsdenii* under conditions of prolonged carbohydrate challenge or an abrupt and extreme increase in dietary starch or soluble fiber to determine its ability to mitigate or eliminate serious, and potentially fatal digestive disturbances. The findings of Leventhal et al. (2011) support the hypothesis that response to *M. elsdenii* will increase with a more severe carbohydrate challenge.

Limitations

Per Brown et al. (2006), great variability exists in susceptibility of cattle to acidosis as well as their ability to cope with abrupt changes in diet. The same is true of horses. Substantial variation was evident over the course of this study in the reaction of individual horses to alterations in their diets and treatment with *M. elsdenii*. Miller (2013) hypothesized that to some

extent, lack of treatment differences may be attributed to this variation, especially in studies utilizing modest animal numbers. Small sample size and great inter-horse variability resulted in high standard errors in this trial, making it difficult to establish statistically significant treatment effects. Statistical power of the results could be improved by increasing sample size in future experiments. The washout period of 28 d may not have been sufficient to prevent bacterial carryover. Due to difficulties with PCR in this study, bacterial carry-over between treatment periods was unable to be evaluated. Although horses had no direct contact with the probiotic culture during the treatment period in which they served as a control, a potential for cross-contamination between treatments within periods exists as horses of different treatments were housed in adjacent stalls. Cross-contamination or carry-over may explain some of the lack of differences between treatment observed in cecal VFA concentrations.

Table 4.1 Treatment assignments randomized within each treatment period

Horse ID	Period 1	Period 2	Period 3
0	Lyophilized powder[3]	Control[1]	Oral drench[2]
1	Control	Oral drench	Lyophilized powder
2	Oral drench	Lyophilized powder	Control
3	Control	Oral drench	Lyophilized powder
4	Oral drench	Control	Lyophilized powder
6	Oral drench	Lyophilized powder	Control
7	Lyophilized powder	Oral drench	Control
10	Lyophilized powder	Control	Oral drench

[1] Horses did not directly receive *M. elsdenii*

[2] Horses received 50 mL *M. elsdenii* as an oral gavage (2.12×10^9 CFU/mL) at the onset of each treatment period

[3] Horses received approximately 0.79 g *M. elsdenii* daily as a lyophilized powder averaging 8.58×10^9 CFU/g within 2 commercially produced equine pill pockets (Uncle Jimmy's Squeezy Buns Pliable Pill Pocket Horse Treats, Uncle Jimmy's, New Castle, PA) administered prior to the morning feeding

Table 4.2 Lyophilized *Megasphaera elsdenii* daily counts (CFU/mL) during treatment periods

			Dilution‡								
			-4		-5		-6				
Period	Day	Weight, g	A	B	A	B	A	B	CFU/mL	CFU/g	Appearance
	1	0.34	TNTC ᵀ	TNTC	45	53	5	7	4.90E+07	5.76E+08	Dark, clumpy
	2	0.35	TNTC	TNTC	TNTC	TNTC	49	44	4.65E+08	5.31E+09	White powder
	3	0.25	TNTC	TNTC	TNTC	TNTC	72	77	7.45E+08	1.19E+10	White powder
	4	0.35	TNTC	TNTC	TNTC	TNTC	37	40	3.85E+08	4.40E+09	White powder
	5	0.42	TNTC	TNTC	TNTC	TNTC	58	62	6.00E+08	5.71E+09	White powder
	6	0.45	TNTC	TNTC	TNTC	TNTC	76	67	7.15E+08	6.36E+09	White powder
1	7	0.42	TNTC	TNTC	TNTC	TNTC	62	58	6.00E+08	5.71E+09	White powder
	1	0.38	TNTC	TNTC	120	99	18	17	1.10E+09	1.16E+10	White powder
	2	0.41	TNTC	TNTC	9	6	1	1	7.50E+07	7.32E+08	White, clumpy
	3	0.44	TNTC	TNTC	37	37	3	4	3.70E+08	3.36E+09	White powder
	5	0.42	30	21	2	4	0	0	2.55E+06	2.43E+07	Brown, clumpy
	6	0.44	158	233	20	29	0	0	1.96E+07	1.78E+08	White, clumpy
2	7	0.47	TNTC	TNTC	TNTC	TNTC	39	33	3.60E+08	3.06E+09	White powder
	1	0.38	TNTC	TNTC	TNTC	TNTC	132	235	1.84E+09	1.94E+10	White powder
	3	0.40	TNTC	TNTC	TNTC	TNTC	183	187	1.85E+09	1.85E+10	White powder
	5	0.37	TNTC	TNTC	TNTC	TNTC	172	167	1.70E+09	1.84E+10	White powder
3*	7	0.38	TNTC	TNTC	TNTC	TNTC	110	103	1.07E+09	1.13E+10	White powder

*During period 3, contents of lyophilized packets from d 2, 4, and 6 rehydrated prior to plating; counts were unobtainable on these days. Packets used to administer *M. elsdenii* to horses were not affected.

ᵀ TNTC = too numerous to count

‡ Samples were rehydrated with 4 mL deionized water, diluted and plated in duplicate.

A = Duplicate 1, B = Duplicate 2 within each dilution

Table 4.3 Dietary nutrient analyses (dry matter basis)

Component, %	Brome Hay 1[a]	Brome Hay 2[b]	Concentrate [c]
DM	90.5	92.0	87.9
CP	8.2	8.4	14.5
ADF	39.2	42.2	8.1
aNDF*	63.9	68.9	15.8
Crude Fat	2.5	2.1	--
Starch	1.6	0.8	--
Ash	7.27	6.93	--
DE, Mcal/kg	2.05	0.87	1.60
Calcium	0.38	0.30	0.87
Phosphorus	0.15	0.22	0.74
Magnesium	0.17	0.16	0.17
Potassium	1.50	1.74	0.88

[a] Fed at a rate of 1% BW AF/d during treatment periods

[b] Fed *ad libitum* during washout

[c] Fed in increasing amounts of 0.2% BW AF/d to a maximum of 1% BW AF during treatment periods

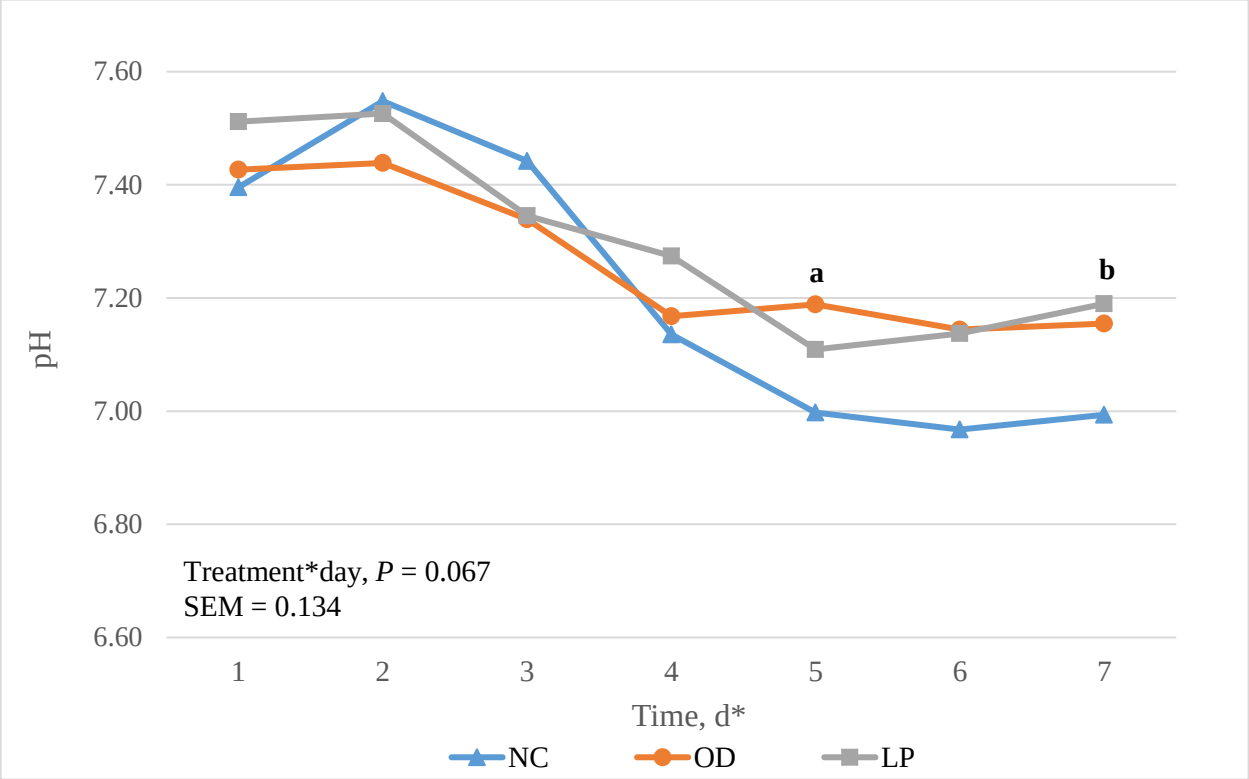
*aNDF solution includes α-amylase

Table 4.4 Composition of equine experimental concentrate [a]

Ingredient, % of diet	Inclusion Level
Corn	20.00
Oats	61.67
Molasses	10.00
Soybean Meal, 48%	5.22
Limestone	1.25
Salt	0.50
Mono calcium phosphate	1.02
Vit A 30,000	0.01
Vit D 30,000	0.00
Vit E 20,000	0.25
Copper sulfate	0.01
Zinc oxide	0.01
Sodium selenite	0.06

[a] Fed during treatment periods in increasing amounts of 0.2% BW AF/d to a maximum of 1% BW AF

Figure 4.1 Effect of *Megasphaera elsdenii* on daily mean cecal pH in the equine



NC - not treated with *M. elsdenii*
OD - horses received 50 mL *M. elsdenii* as an oral gavage (2.12 x 10^9 CFU/mL) at the onset of each treatment period
LP - horses received approximately 0.79 g *M. elsdenii* daily as a lyophilized powder averaging 8.58 x 10^9 CFU/g contained within 2 commercially produced equine pill pockets, administered prior to the morning feeding
[a] OD tends to be different from NC at $P < 0.10$
[b] LP tends to be different from NC at $P < 0.10$
*Grain inclusion increased at a rate of 0.2% BW AF/d to a maximum of 1% BW AF fed on d 5 through d 7

Table 4.5 Effects of *Megasphaera elsdenii* on equine daily mean cecal VFA concentrations

						P-value	
Item*	**Day**	**NC[1]**	**OD[2]**	**LP[3]**	**SEM**	**Treatment ᵀ**	**Contrast ᵀᵀ**
	0	47.08 $^{A, a}$	43.29 $^{A, ab}$	46.86 $^{A, a}$			
	5	43.78 $^{A, a}$	40.84 $^{A, b}$	40.33 $^{A, ac}$			
	6	39.47 $^{A, b}$	38.49 $^{A, ab}$	36.71 $^{A, b}$			
Acetate	7	38.42 $^{A, b}$	37.48 $^{A, a}$	38.34 $^{A, bc}$	5.861	D	0.59
	0	14.90 $^{A, a}$	14.25 $^{A, a}$	16.32 $^{A, ab}$			
	5	19.26 $^{A, b}$	18.78 $^{A, b}$	18.36 $^{A, a}$			
	6	17.87 $^{A, abc}$	17.66 $^{A, ab}$	15.61 $^{A, b}$			
Propionate	7	16.81 $^{A, ac}$	15.67 $^{A, a}$	15.42 $^{A, b}$	3.083	D	0.59
	0	3.51 $^{A, a}$	3.32 $^{A, a}$	3.39 $^{A, a}$			
	5	2.37 $^{A, b}$	2.32 $^{A, b}$	2.31 $^{A, b}$			
	6	2.33 $^{A, b}$	2.29 $^{A, b}$	2.44 $^{A, b}$			
A:P	7	2.42 $^{A, b}$	2.50 $^{A, c}$	2.61 $^{B, c}$	0.181	D	0.89
	0	5.11 $^{A, ab}$	4.28 $^{A, ab}$	4.70 $^{A, a}$			
	5	4.70 $^{A, b}$	4.17 $^{A, a}$	4.25 $^{A, a}$			
	6	4.16 $^{A, a}$	3.67 $^{A, ab}$	3.93 $^{A, a}$			
Butyrate	7	3.54 $^{A, c}$	3.42 $^{A, b}$	3.78 $^{A, a}$	0.900	D	0.45
	0	0.000 $^{A, a}$	0.000 $^{A, ab}$	0.120 $^{B, a}$			
	5	0.013 $^{A, a}$	0.019 $^{A, b}$	0.071 $^{A, a}$			
	6	0.003 $^{A, a}$	0.051 $^{A, ab}$	0.032 $^{A, b}$			
Isobutyrate	7	0.003 $^{A, a}$	0.054 $^{A, a}$	0.035 $^{A, b}$	0.0516	I	0.25
	0	0.162 $^{A, a}$	0.036 $^{A, a}$	0.083 $^{A, a}$			
	5	0.134 $^{A, a}$	0.046 $^{B, a}$	0.081 $^{AB, a}$			
	6	0.114 $^{A, a}$	0.063 $^{A, a}$	0.104 $^{A, a}$			
Valerate	7	0.033 $^{A, b}$	0.59 $^{AB, a}$	0.123 $^{B, a}$	0.0955	I	0.29
	0	0.000 $^{A, a}$	0.000 $^{A, ab}$	0.135 $^{B, a}$			
	5	0.013 $^{A, a}$	0.019 $^{A, b}$	0.078 $^{A, ab}$			
	6	0.004 $^{A, a}$	0.052 $^{A, ab}$	0.045 $^{A, bc}$			
Isovalerate	7	0.004 $^{A, a}$	0.057 $^{A, a}$	0.036 $^{A, c}$	0.0570	I	0.25
	0	0.000 $^{A, a}$	0.000 $^{A, a}$	0.007 $^{A, ab}$			
	5	0.000 $^{A, a}$	0.000 $^{A, a}$	0.000 $^{A, a}$			
	6	0.000 $^{A, a}$	0.000 $^{A, a}$	0.007 $^{A, ab}$			
Caproate	7	0.000 $^{A, a}$	0.000 $^{A, a}$	0.016 $^{B, b}$	0.0121	--	0.55
	0	67.23 $^{A, ab}$	61.87 $^{A, ab}$	68.18 $^{A, a}$			
	5	67.91 $^{A, a}$	63.90 $^{A, b}$	63.13 $^{A, a}$			
	6	61.62 $^{A, b}$	60.00 $^{A, ab}$	56.39 $^{A, b}$			
Total VFA	7	58.82 $^{A, b}$	56.76 $^{A, a}$	57.72 $^{A, ab}$	9.432	D	0.58

*All VFA concentration reported in mM

[1] NC - horses did not directly receive *M. elsdenii*

[2] OD - horses received 50 mL *M. elsdenii* as an oral gavage (2.12×10^9 CFU/mL) at the onset of each treatment period

[3] LP - horses received approximately 0.79 g *M. elsdenii* daily as a freeze-dried powder averaging 8.58×10^9 CFU/g contained within 2 commercially produced equine pill pockets, administered prior to the morning feeding

ᵀ D = Effect of day of sampling; I = Interaction between treatment and day of sampling; $P \leq 0.05$

ᵀᵀ Contrast *M. elsdenii* vs. control

$^{A, B}$ Means within a row without a common superscript are different at $P \leq 0.05$

$^{a, b}$ Means within a column without a common superscript are different at $P \leq 0.05$

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