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STRATEGIC NUTRITIONAL INTERVENTIONS WITH PROBIOTICS AND THEIR RELATIONSHIP TO PERFORMANCE, FEEDING BEHAVIOR, AND RETICULORUMEN ENVIRONMENT IN COWS AND CALVES UNDER SUBACUTE RUMINAL ACIDOSIS RISK

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food, and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

STRATEGIC NUTRITIONAL INTERVENTIONS WITH PROBIOTICS AND THEIR RELATIONSHIP TO PERFORMANCE, FEEDING BEHAVIOR, AND RETICULORUMEN ENVIRONMENT IN COWS AND CALVES UNDER SUBACUTE RUMINAL ACIDOSIS RISK

Subacute ruminal acidosis (SARA) is a digestive disorder in ruminants characterized by extended periods of low reticulorumen pH. This digestive disorder is commonly observed in ruminants fed diets with elevated proportions of non-fibrous carbohydrates. This disorder has been vastly studied in adult dairy cattle and has been associated with losses in milk production, changes in feeding behavior, damage to the gastrointestinal tract, and premature culling. Although vastly studied in adult cattle, there is limited research on the effects of SARA in calves. SARA might be prevented by modifying the reticulorumen environment with probiotic supplements containing lactate-utilizing bacteria such as *Megasphaera elsdenii*. *Megasphaera elsdenii* is a naturally occurring rumen microorganism known for stabilizing ruminal pH. Thus, the objectives of this dissertation were to investigate the effects of *Megasphaera elsdenii* supplementation strategies to prevent SARA in cows and calves in commercial situations, therefore improving animal welfare and performance. The first objective of this dissertation was to investigate the effects of *Megasphaera elsdenii* supplementation via oral drench on reticulorumen pH, milk production, and feed intake and behavior of lactating cows undergoing a ruminal acidosis challenge. The second objective of this dissertation was to evaluate the effects of early-life *Megasphaera elsdenii* supplementation on feed intake, performance, and feeding behavior patterns of dairy-beef crossbred calves. Lastly, the third objective of this dissertation was to evaluate how early-life *Megasphaera elsdenii* supplementation with an oral probiotic capsule affects the reticulorumen fermentation and development in dairy-beef crossbred calves. Overall strategic nutritional interventions with *Megasphaera elsdenii* supplementation were beneficial for cows and calves under SARA risk. As cows receiving the probiotic experienced shorter and less intense SARA when compared to control cows during a SARA challenge. Furthermore, calves supplemented with *Megasphaera elsdenii* at early production stages displayed greater feed intake, weight gain, and superior reticulorumen development when compared to control calves. Future research should investigate the effects of *Megasphaera elsdenii* supplementation on the reticulorumen microbiome of cows and calves under SARA risk.

KEYWORDS: Ruminal acidosis, *Megasphaera elsdenii*, feeding strategy, rumen development

Gustavo Mazon Correa Alves

12/08/2023

Date

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. REVIEW OF LITERATURE

1.1 INTRODUCTION

Dairy cow productivity in the United States (NASS, 2023) and Europe (FAS, 2023) has been steadily increasing during recent years. This increase in productivity is associated with an increase in the nutrient requirements by dairy cattle (NASEM, 2021). However, cows might not be able to fully fulfill their increased nutrient requirements by increasing intake, as gut fill is one of the many factors that limit dry matter intake (DMI) in cattle (Allen, 1996). Thus, a common nutritional strategy to fulfill the increased nutrient requirements without limiting DMI caused by gut fill, is to increase the nutrient density of the diets by offering greater proportions of non-fibrous carbohydrates (Allen, 2000, Elmhadi et al., 2022).

Diets containing greater proportions of non-fibrous carbohydrates favor the production of volatile fatty acids (VFA) and lactate in the reticulorumen (Nafikov and Beitz, 2007). Furthermore, these carbohydrate-rich diets favor the production of propionate (Khan et al., 2011b), one of the main glucose precursors in ruminants, specially lactating dairy cows (Nafikov and Beitz, 2007). However, the accumulation of VFA and lactate in the reticulorumen lowers its pH (Nagaraja and Titgemeyer, 2007a), increasing the risks of acute and subacute reticulorumen acidosis (SARA(Krause and Oetzel, 2006a, Khorrami et al., 2021).

Subacute reticulorumen acidosis is a common digestive disorder in dairy cattle characterized by long periods of time with reticulorumen pH between 5.5 and 5.0 (Gozho et al., 2007, Plaizier et al., 2008, Jaramillo-López et al., 2017). Whereas acute

reticulorumen acidosis is characterized by abrupt drops in reticulorumen pH below 5.0 followed by clinical symptoms such as ataxia, diarrhea, reduced reticulorumen motility, and even death (Snyder and Credille, 2017). Subacute reticulorumen acidosis can affect the reticulorumen papillae structure (Steele et al., 2011), reduce milk yield (Colman et al., 2010), reduce milk fat percentage (Colman et al., 2010, Golder et al., 2023), affect feeding behavior (DeVries et al., 2008, DeVries et al., 2009, Coon et al., 2019), and other secondary effects are being study recently. The association of SARA with severe implications such as liver abscesses (Nagaraja and Titgemeyer, 2007a, Elmhadi et al., 2022) and laminitis (Passos et al., 2023) are established; thus despite the lack of apparent clinical signs, SARA is a costly disease for farmers (Rojo-Gimeno et al., 2018).

Despite being vastly studied and reviewed (Krause and Oetzel, 2006a, Monteiro and Faciola, 2020, Plaizier et al., 2022) in adult cattle, there is limited information about SARA in calves. Previous research has shown that reticulorumen pH in calves is consistently lower than in adult cattle (Gentile et al., 2004, Suarez-Mena et al., 2016, Yohe et al., 2018), yet calves are still prone to decreases in reticulorumen pH, especially during weaning (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020). Recent studies reported that calves experiencing feed induced SARA have displayed reduced solid feed intake, reduced growth, and poor reticulorumen development when compared to their control counterparts (Li et al., 2019, Gelsinger et al., 2020).

There is a myriad of acute and SARA treatment and prevention methods in cattle. However, most research work focuses on the prevention of the disorder as treatment might be labor intensive and mostly recommended for severe acute reticulorumen acidosis cases (Snyder and Credille, 2017). Some of the many SARA prevention methods include

adjusting the physically effective fiber levels in the diet (Yang and Beauchemin, 2006, Maulfair et al., 2013, Khorrami et al., 2021), dietary inclusion of buffers (Enemark, 2008, Srivastava et al., 2021), dietary inclusion of ionophores (Mutsvangwa et al., 2002, Pacheco et al., 2023), provision of free-choice buffers (Keunen et al., 2003, Paton et al., 2006, Krause et al., 2009) or free choice straw (Genís et al., 2021, Monjezi et al., 2022).

The vast adoption of antimicrobials in livestock diets has been associated to increases in antimicrobial resistance, which is a risk both for animal and human health (Rousham et al., 2018, Garcia et al., 2019). Thus, there is a need to develop new alternatives to dietary antimicrobial use. In fact, following the prohibition of ionophores as feed additives for cattle, such as monensin in Europe (EuropeanCommision, 2007), researchers started investigating natural alternatives for SARA management such as probiotics (Cangiano et al., 2020, Barreto et al., 2021). Probiotics are defined as live strains of select microorganisms which might grant a health benefit to the host (Markowiak and Śliżewska, 2017). When developing a probiotic it is essential that the strains chosen are safe for human and animals, specific for a target disorder, and that they can survive in the gastrointestinal tract (Anadón et al., 2006). Multi-species probiotics seem to have a positive effect on performance and health when animals are under stress (Cangiano et al., 2020). Yet, we must acknowledge that the reticulorumen is a complex and dynamic environment where multiple bacteria compete for substrates (Weimer, 2015, Costa-Roura et al., 2022, Du et al., 2023). One microorganism that has great probiotic potential for animals under SARA risk is the lactate-utilizing bacteria *Megasphaera elsdenii.*

Megasphaera elsdenii is a gram-negative rumen microorganism known for metabolizing lactic acid and helping to stabilize ruminal pH (Counotte et al., 1981a,

Nocek, 1997, Nagaraja and Titgemeyer, 2007a). It can ferment lactic acid to acetic and propionic acids (Hino et al., 1994). Some strains of *Megasphaera elsdenii* have been patented and are now available to be used as SARA control tools (Leedle et al., 1990, Horn et al., Inventors. 2009). Hence, an opportunity exists for implementing strategic *Megasphaera elsdenii* supplementation for cows and calves under SARA risk.

The primary aim of this review is to highlight the causes, consequences, and prevention strategies of SARA in both cows and calves. First, this review will summarize the development and differentiation of the reticulorumen and its microbiome from birth to adulthood to set the necessary background to understand the environment and circumstances in which SARA may arise. The next portion of this review will focus on defining and exploring the causes and consequences of SARA in cows and calves. Finally, this review will cover the existing and potential nutritional management strategies that can be adopted to prevent or mitigate the effects of this nutritional disorder, such as adjusting the levels of non-fibrous carbohydrates in the diet, supplementation with dietary buffers, or probiotics. This review aims to emphasize the use of probiotics to prevent SARA, specifically focusing on the current research available on *Megasphaera elsdenii* supplementation to cows and calves under SARA risk.

1.2 THE RETICULORUMEN DEVELOPMENT AND FUNCTION

A fully developed and functional reticulorumen is fundamental for optimal feed digestion, health, and production in cattle (Baldwin and Connor, 2017). However, newborn calves do not have a functional reticulorumen and are considered functional monogastrics as milk (or milk replacer) is digested and absorbed in the abomasum and small intestine,

respectively (Baldwin et al., 2004, Meale et al., 2017a). To achieve full development, the reticulorumen must go through three main steps during the rearing period: i) microbial colonization, ii) functional development (increases in fermentation capacity and absorption), and iii) anatomical development (increases in reticulorumen and papillae size) (Yáñez-Ruiz et al., 2015). These changes are necessary for calves to develop into mature ruminants with an established symbiotic relationship with microorganisms in their forestomaches in order to digest plant cell walls and contents via fermentation, differentiating themselves from monogastric animals feeding strategies (Russell, 2002).

This section of the literature review will first describe the reticulorumen microorganisms. Then, we will focus on the microbial, metabolic, and anatomical development of the reticulorumen in calves from birth to postweaning and highlight the research investigating nutritional and management practices that can be adopted during this period to ensure proper reticulorumen development in calves.

1.2.1 *Microbiome colonization in the calf*

The development of a functional microbiome in calves is critical to produce VFA in the reticulorumen aiding calves in the transition from liquid to solid diets. The calf reticulorumen microbiome is naïve at birth and it can be affected by several factors such as genetics (Paz et al., 2016), environment (O'Hara et al., 2020), age (Jami et al., 2013), the diet (Dias et al., 2018, Dill-McFarland et al., 2019), and management practices (Amin et al., 2021). Thus, in this section we will discuss the development of the calf microbiome from *in utero* to weaning as well as its relationship to environmental and management factors.

1.2.1.1 The calf microbiome at birth.

It was believed that the gastrointestinal tract in calves *in utero* was sterile and microbial colonization started during birth following the rupture of the amniotic membranes (Arshad et al., 2021). Those beliefs were mostly based on fetus sterility observations from the human literature (Mackie et al., 1999, Malmuthuge and Griebel, 2018, Kennedy et al., 2021). However, in a rigorously controlled study, Guzman et al. (2020) reported that viable bacteria and archaea were detected in the reticulorumen fluid and tissue of calf fetuses from 5 to 7 months of gestation. Furthermore, the authors reported that the majority of the bacteria detected in the reticulorumen fluid or tissue of calf fetuses were from aerobic or facultative anaerobic microorganisms (Guzman et al., 2020). Despite the presence of these microorganisms *in utero,* the microbiome in neonatal calves lacks in diversity and changes rapidly after birth as the population of anaerobic and facultative anaerobic microorganisms is replaced by anaerobic microorganisms (Du et al., 2023). In fact, shortly after birth, the calf reticulorumen displays a few microorganisms commonly observed in adult cattle. For example, Jami et al. (2013) utilized quantitative real-time PCR analysis and reported the presence of fibrolytic (e.g. *Ruminococcus albus)*, amylolytic (e.g. *Streptococcus bovis*), and lactate-utilizing (e.g. *Selenomonas ruminantium* and *Megasphaera elsdenii*) bacteria in the reticulorumen of calves between 1 and 3 days of age. The persistence of early colonizing reticulorumen bacteria from birth to adulthood has also been recently confirmed in a study by Furman et al. (2020).

The calf microbiome is dynamic and displays constant changes as the calf ages (Jami et al., 2013) .As they age, calves display a natural and gradual increase in solid feed

intake, which brings new substrates into the reticulorumen changing microbial populations to show specialization towards those substrates (Rey et al., 2014, Meale et al., 2017a). As the microbiome in calves becomes more specialized in solid feed fermentation, it starts to resemble the microbiome observed in adult cattle. In fact, Jami et al. (2013) reported that at 2 months of age the calf microbiome starts to resemble the adult microbiome and that this similarity only increases with age. During the microbial colonization and stabilization process, it can be affected by several factors such as environment (O'Hara et al., 2020), management practices (Amin et al., 2021), and diet (Dias et al., 2018, Dill-McFarland et al., 2019). Thus, the following section will focus on the effects of environment and management practices on the calf microbiome.

1.2.1.2 Effects of environment and management on the calf microbiome

Farm environment and management practices varies greatly around the world (Hötzel et al., 2014, Staněk et al., 2014, Medrano-Galarza et al., 2017). Thus, it is important to understand how different management practices might affect microbial populations during the reticulorumen development phase in early life.

One of the first effects of environment and management on microbiome development in calves happens at birth. Furman et al. (2020) reported that reticulorumen microbiome of calves delivered via C-section displayed greater microbial diversity and was less stable over time when compared to calves delivered via vaginal birth. In addition, calf rearing environment might affect the early-life reticulorumen microbiome. In a study investigating the effect of rearing environment on reticulorumen microbiome in beef calves, O'Hara et al. (2020) reported that calves born on different farms presented different

reticulorumen microbiomes in the first month of life despite being reared under similar management and dietary conditions.

Calf rearing and management practices might also affect the early-life gut microbiome and development. For example, Malmuthuge et al. (2015) reported that calves fed pasteurized colostrum had lower counts of *Escherichia coli* and greater counts of *Bifidobacterium* in their lower gut when compared to calves fed unpasteurized colostrum. Furthermore, other management factors such as weaning age and method have an impact on the reticulorumen microbiome in calves. In a study investigating the effects of weaning age on the reticulorumen microbiome in dairy calves, Meale et al. (2017b) reported that calves weaned at 6 weeks of age displayed more drastic shifts and less microbiome resilience when compared to calves weaned at 8 weeks of age. Similarly, Amin et al. (2021) reported drastic changes in bacterial diversity in the reticulorumen of dairy calves weaned at 7 weeks of age. In addition, the authors reported that the reticulorumen microbiome of calves weaned at 7 weeks of age displayed lower counts of amylolytic bacteria and increased fibrolytic bacteria when compared to calves weaned at 17 weeks of age. Still, weaning calves at 17 weeks of age does not reflect current dairy calf raising practices (Urie et al., 2018). Authors agree that delaying the age of weaning and allows calves to adapt to the ingestion of solid feeds promoting a gradual shift in the reticulorumen microbiome (Meale et al., 2017b, Amin et al., 2021). However, diet is one of the major factors associated with changes in the calf microbiome. Thus, the following section will explore the effects of diet in the calf microbiome.

1.2.1.3 Effects of diet on the calf microbiome

Calves are exposed to different diets during the rearing period. One of the most drastic dietary changes happens during weaning as the calf is transitioning from a liquid to solid diet. To understand the effects of dietary composition on reticulorumen microbial community, it is important to understand the microorganisms themselves. Thus, in this section we will describe the most common reticulorumen microorganisms and describe how they might be affected by dietary composition.

The reticulorumen is a mainly anaerobic environment, and its microbiome is composed of bacteria, protozoa, fungi, and viruses (Russell, 2002, Gruninger et al., 2019, Newbold and Ramos-Morales, 2020). Given the microbial diversity and the complexity of their interactions, researchers tend to divide the reticulorumen microbiome into functional groups (e.g. fibrolytic, amylolytic, lactate-utilizers, and hydrogen-utilizers) based on their substrates or products (Moraïs and Mizrahi, 2019). The fibrolytic group is composed by bacteria *(e.g. Ruminococcus albus*, *Fibrobacter succionogenes*, and *Butyvibrio fibrosolvens*), protozoa (e.g. *Polyplastron*), and fungi with their primary substrate being cellulose and products being acetate, formate, and hydrogen (Russell, 2002, Newbold et al., 2015, Moraïs and Mizrahi, 2019). The amylolytic group is composed mostly by bacteria (e.g. *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis*) with their primary substrate being starch and sugars and their products include acetate, formate, lactate, and propionate (Russell, 2002, Moraïs and Mizrahi, 2019). Lactate-utilizers are bacteria which main substrate is mainly lactate and their products include acetate, propionate, butyrate, hydrogen, and even lactate depending on the substrate utilized (e.g. *Megasphaera elsdenii*, *Selenomonas ruminantium*; (Russell, 2002, Moraïs and Mizrahi, 2019). Hydrogen-utilizing are mainly archaea which main substrates are hydrogen and

carbon dioxide and their main product is methane (e.g. *Methanobrevibacter ruminantium*, *Methanosarcina barkeri*; (Russell, 2002, Moraïs and Mizrahi, 2019).

Common reticulorumen microorganisms show different substrate preferences, thus the abundance of those substrates in the reticulorumen will influence how each microbial population grows. For example, forages are rich in structural carbohydrates such as cellulose and hemicellulose. Consequently, forage ingestion has been associated with increases in reticulorumen cellulolytic bacteria and protozoa populations (Suárez et al., 2006b, Terré et al., 2013b, Khan et al., 2016). On the other hand, calf starters (also referred to in the literature as grain or concentrate) are rich in non-fibrous carbohydrates such as starch and sugars. Hence, calf starter intake have been associated with decreases in reticulorumen lactose fermenting bacteria and increases in amylolytic and lactate utilizing bacteria (Terré et al., 2013b, Khan et al., 2016, Dias et al., 2018).

Differences in the inclusion rate of forages or non-fibrous carbohydrates will affect the reticulorumen microbiome and, consequently, affect reticulorumen VFA production. Thus, the next session will explore how different dietary and management strategies can affect reticulorumen VFA production and their association with reticulorumen anatomical growth.

1.2.2 *Reticulorumen functional and anatomical development*

The capacity to produce and absorb VFA are extremely important for the functional and anatomical development of the reticulorumen, being directly associated with calf growth. In fact, reticulorumen fermentation and VFA production becomes fundamental with age, as the calf's energy metabolisms starts to focus mostly on VFA utilization (Klotz

and Heitmann, 2007, Guilloteaul et al., 2009). To utilize the VFA produced in the reticulorumen, the animal must absorb it via the reticulorumen epithelium (Grünberg and Constable, 2008, Morgavi et al., 2013). Yet, milk tends to bypass the rumen via the esophageal groove therefore, the production of VFA in the reticulorumen is highly associated with the ingestion of solid feeds (Baldwin et al., 2004, Meale et al., 2017a). The next section of this review will explore the association between diet composition, VFA production, and reticulorumen functional and anatomical development.

1.2.2.1 Effects of solid diet on reticulorumen functional and anatomical development

The ingestion of solid diets is fundamental for VFA production in the reticulorumen. In addition, the VFA profile is extremely important for the functional and anatomical development of the reticulorumen and calf growth (Terré et al., 2013b). The production of VFA by the reticulorumen microorganisms provides the chemical stimulus needed for papillae growth and VFA absorption capacity (Sander et al., 1959, Tamate et al., 1962, Diao et al., 2019). In fact, Nishihara et al. (2023) reported that increased VFA concentrations were associated with increased expression in genes associated with VFA absorption in the reticulorumen epithelium. Thus, the production and profile of VFA is extremely important for the functional and anatomical development of the reticulorumen and calf growth.

Ingestion of calf starters containing non-fibrous carbohydrates have been associated with increased production of butyrate and propionate (Terré et al., 2013b, Khan et al., 2016, Dias et al., 2018). Whereas forage-based diets have been mostly associated with increases in reticulorumen acetate production (Terré et al., 2013b, Khan et al., 2016,

Dias et al., 2018). However, the ability to stimulate reticulorumen functional and anatomical development in calves differs between VFA. Increases in papillae dimensions and VFA absorption capacity have been associated with the concentrations of butyrate followed by acetate, and propionate, respectively (Bergman, 1990). Tamate et al. (1962) demonstrated the role VFA and solid feed intake played in reticulorumen development in a trial. The authors reported that calves receiving milk and a reticulorumen infusion of propionate and butyrate had similar reticulorumen papillae dimensions compared to calves with access to milk, hay, and calf starter. However, authors reported that the reticulorumen weights of calves receiving milk and the VFA infusion were lower than the reticulorumen weights of calves with access to milk, hay, and calf starter (Tamate et al., 1962). The physical bulk caused by the presence of solid feed in the reticulorumen aids the development of the reticulorumen musculature being associated with greater reticulorumen weights and capacity (Tamate et al., 1962, Khan et al., 2008, Khan et al., 2011a). Therefore, provision of calf starter and forages is recommended to stimulate the production and absorption of VFA by increasing reticulorumen musculature and papillae development in calves (Terré et al., 2013b). However, low solid feed intake has been associated with low VFA production and poor reticulorumen functional and anatomical development.

Management practices such as providing calves with high milk allowances might hinder solid feed intake and affect reticulorumen development. Feeding calves higher milk allowances has a positive effect on weight gain and reduces the display of behaviors associated with hunger (Rosenberger et al., 2017). However, calves fed greater amounts of milk display lower solid feed intake, especially during the preweaning period (Kristensen et al., 2007, Rosenberger et al., 2017). In a study feeding calves different milk allowances

from two to five weeks of age, reported that as milk allowance increased, solid feed intake and reticulorumen butyrate concentrations decreased (Kristensen et al., 2007). The same authors harvested the calves at five weeks of age and reported that reticulorumen weight decreased as milk allowance increased (Kristensen et al., 2007). Lastly, low solid feed intake during the preweaning period is associated with poor growth during the post weaning period (Nielsen et al., 2008, Sweeney et al., 2010). Therefore, it is important to adopt strategies that promote solid feed intake and VFA production during the preweaning period. Thus, the next section will focus on management strategies that focus on increasing solid feed intake during the preweaning period.

1.2.2.2 Promoting solid feed intake during the preweaning period

Promoting solid feed intake in early life is necessary for proper reticulorumen development and for guaranteeing calf growth during the weaning period. The transition from liquid to solid diets can be stressful for calves. However, the adoption of proper feeding management strategies can ensure calf success during the weaning and postweaning periods and ameliorate the stress of this transition period.

Low solid feed intake during the preweaning period has been associated with high milk allowances (Kristensen et al., 2007). For instance, when calves fed larger amounts of milk are abruptly weaned, they display decreased solid feed intake, poor weight gain, and display increased signs of hunger during weaning (Nielsen et al., 2008, Sweeney et al., 2010).

Gradual (or step-down) weaning programs utilize a gradual decrease in the amount of milk offered to calves as weaning age approaches. The adoption of gradual weaning

programs has been used to ease the transition from liquid to solid diets and promote solid feed intake during the preweaning period. In a trial comparing abrupt and gradual weaned Holstein heifers, Khan et al. (2007a) reported that heifers undergoing a step-down weaning program displayed greater solid feed dry matter intake, body weight gain, and feed efficiency compared to abruptly weaned heifers. Then, in a similar study with male Holstein calves, Khan et al. (2007b) reported that calves managed under a step-down weaning program displayed greater solid feed intake and greater body weight gain during the pre and postweaning periods compared to abruptly weaned calves. Furthermore, the authors reported that calves managed under a step-down weaning program displayed greater reticulorumen acetate, propionate, butyrate, and total VFA concentrations at weaning and postweaning compared to abruptly weaned calves. Lastly, the same study reported that the greater solid feed intake and VFA concentration displayed by gradually weaned calves was responsible for these calves displaying greater reticulorumen size, papillae size, and papillae concentration compared to abruptly weaned calves (Khan et al., 2007b).

Solid feed intake is fundamental to stimulate reticulorumen microbial growth, VFA production, and reticulorumen development in calves. The adoption of management practices that promote solid feed intake is necessary to ensure proper reticulorumen development and ensure calf success postweaning. In fact, the reticulorumen microbiome and VFA concentration in postweaning calves display similarities to what is observed in adult cows (Suárez et al. (2006a). Thus, in the next section we will briefly describe the reticulorumen environment in the adult cow and describe how it can be affected by different nutritional and management practices.

1.2.3 *The reticulorumen of an adult cow*

A healthy and stable reticulorumen environment is needed for optimal feed digestibility, health, and production in adult dairy cows. The cow reticulorumen microbiome allows them to digest feeds that are unsuitable for human nutrition into high-value protein sources for humans such as milk and meat (Russell, 2002). Thus, in this section we will describe the unique characteristics of the adult cattle microbiome as well as how it might be affected by diet.

The adult cow microbiome displays a degree in microbial specialization for feed digestion that is reflected by the reduced microbial diversity in comparison to calves. In fact, it is estimated that over 90% of the microbial population in the adult cow is composed of bacteria and protozoa (Weimer, 2015). In fact, a study evaluating the microbiome of ruminants across 35 countries, Henderson et al. (2015) reported that animals share a common core bacterial microbiome. The authors also reported that although distribution of species might vary across individual, the core reticulorumen bacteria genus observed across animals were *Prevotella*, *Butyvibrio*, and *Ruminococcus.* More recently, the presence of a shared core microbiome was also observed across 1,016 European dairy cows despite differences location, breed, and diet (Wallace et al., 2019). The presence of a core microbial community does not imply lack of microbial diversity in the reticulorumen. For example, the Hungate1000, a community effort aiming to catalog reticulorumen bacteria and archaea, has 501 catalogued and cultured species (Seshadri et al., 2018). On the other hand, utilization of the 16S Rrna gene sequences has allowed researchers to catalog more than 13,000 bacterial and 3,500 archaea species that were present in the reticulorumen of

cattle (Kim et al., 2011). Yet, authors still report the presence of unclassified reticulorumen microorganisms (Smith et al., 2022, Zhang et al., 2022).

The microbial diversity observed in the reticulorumen microbiome of cows allows the microbial population to demonstrate redundancy. Redundancy is defined as the overlap in function across multiple microbial species (Weimer, 2015). The combination of a core microbiome and presence of overlapping functions across microbial species makes the reticulorumen of adult cattle to be a highly resilient environment (Weimer, 2015). Westman (1978) defines an ecosystem as resilient based on its ability to restore its structure following disturbances. Later, Weimer et al. (2010) demonstrated the resilience of the reticulorumen environment by performing a total exchange of reticulorumen contents between animals. The authors reported that the reticulorumen microbiome was able to return to its original composition following total exchange of contents (Weimer et al., 2010). Despite demonstrating high resilience, the reticulorumen environment in dairy cows is still subject to changes based mainly on dietary composition (Henderson et al., 2015, Deusch et al., 2017). Thus, the next section will describe the effects of diet on the cow microbiome composition.

1.2.3.1 Effects of diet on cow reticulorumen microbiome and VFA production

Diet composition and management is one of the main factors associated with cattle production and health. Thus, in this section we will describe the effects of dietary composition and management on the reticulorumen microbiome and VFA production in cows.

A comprehensive survey of cattle across 35 countries reported that the microbial communities differed between animals fed forage, mixed (50-70% forage + grain), and grain-based diets (Henderson et al. (2015). In forage fed cattle, the reticulorumen microbiome has greater counts of fibrolytic bacteria compared to other functional groups. On the other hand, cattle fed grain-based diets showed decreases in fibrolytic bacteria and increases in propionate-producing bacteria. Lastly, cattle fed mixed diets presented a microbiome that was an intermediate between forage and grain-fed cattle (Henderson et al., 2015). Similar findings were reported by Zhang et al. (2017) in a trial investigating different forage to grain ratios. The authors reported that the relative abundance of cellulolytic bacteria decreased as dietary non-fibrous carbohydrate inclusion increased. Furthermore, they reported an increase in amylolytic bacteria as grain inclusion increase, which culminated in increases in propionate and butyrate production (Zhang et al., 2017). Wang et al. (2020) also reported similar shifts in reticulorumen microbiome and VFA concentration when comparing cows fed high (70% inclusion) and low (35% inclusion) forage diet. In addition, the authors reported that cows fed low forage diets displayed increases in lactate utilizing bacteria populations compared to cows fed a high forage diet (Wang et al., 2020).

Elevated proportions of non-fibrous carbohydrates in the diet can also affect the reticulorumen environment in lactating dairy cows. For example, in a study feeding isonitrogenous and isoenergetic diets that differed in the level of non-fibrous carbohydrates (NFC), Rustomo et al. (2006) reported that cows fed high NFC diet tended to display lower reticulorumen pH for longer period of time compared to control. The physical form and processing of grains also seems to affect the reticulorumen environment in dairy cows. The

inclusion of processed grains (rolled, steamed, finely ground) facilitates microbial digestion, however it has been associated with decreases in reticulorumen pH in adult cattle (Maulfair et al., 2011, Carvalho and Felix, 2020, Oba and Kammes-Main, 2023). On the other hand, feeding chopped versus ground forages had a positive effect on reticulorumen pH but was associated with lower total VFA concentration in dairy cows (Yang et al., 2001, Beauchemin et al., 2003). However, feeding long forage particle sizes might cause cows to sort against those long particles and ingest mostly finer components of the diet, which can negatively affect reticulorumen pH (Coon et al., 2019).

Modification of dietary forage and grain proportions as well as managing dietary factors such as grain processing and dietary particle size also seems to affect the reticulorumen environment. The importance of dietary management in adult dairy cattle seems to be further highlighted at the beginning of lactation. Upon parturition, cows experience a drastic increase in energy requirements to sustain milk production (NASEM, 2021). In addition, early lactation cows display decreased dry matter intake (Ospina et al., 2010). This reduction in dry matter intake is complex to be explained and it seems to be caused by a multitude of factors such as decreased rumen volume (Reynolds et al., 2004), hormonal (Pushpakumara et al., 2003) and management changes (Proudfoot et al., 2009, Garnsworthy and Topps, 2010). As a strategy to increase energy intake after parturition, nutritionists tend to elevate the nutrient density of postpartum diets by feeding diets containing elevated proportions of non-fibrous carbohydrates, which might lead to decreases in reticulorumen pH and possibly SARA. Thus, in the next section of this review, we will describe the causes, consequences, and prevention strategies of SARA in cows and calves.

1.3 SUBACUTE RETICULORUMEN ACIDOSIS

A stable reticulorumen pH is a critical factor for the maintenance of a healthy microbiome and effective feed digestion. In fact, reticulorumen pH is a combination of factors: i) VFA production, ii) VFA absorption, and iii) buffering. Any drastic disturbances in any of those factors might lead to SARA. Hence, in this section we will define SARA, explore the consequences of the disorder in calves and cows, and describe common prevention methods.

1.3.1 *SARA Definition*

Subacute reticulorumen acidosis is a common digestive disorder observed in dairy and beef cattle characterized by low reticulorumen pH that can affect animal production, behavior, and health (González et al., 2012, Plaizier et al., 2022).

The display of clear visual SARA symptoms is highly variable between individuals and that common symptoms (e.g. decreased feed intake and decreased performance) be strongly associated with other disorders (Wetzels et al., 2017, Plaizier et al., 2018, Plaizier et al., 2022), which makes SARA difficult to be easily diagnosed in the field without a multifactorial approach. The lack of an easy on-farm diagnostics tool might contribute to the relatively elevated prevalence of SARA in dairy herds across the globe. In a cross sectional study across Wisconsin dairy farms, Garrett et al. (1997) reported high SARA prevalence in early and mid-lactation cows. In a study evaluating 315 cows across 26 German dairy herds, Kleen et al. (2013) reported that a cows displaying low reticulorumen pH was elevated. The combination of the lack of an easy diagnostics tool, lack of clear visual symptoms, and the relatively high prevalence, makes SARA a costly disorder for dairy farmers.

Most SARA definitions are based on pH thresholds; therefore, if reticulorumen pH drops below a certain threshold, the animal is considered to be under SARA risk (Plaizier et al., 2022). In grain-adapted dairy cows, normal reticulorumen pH ranges from 5.5 to 6.5 (Nagaraja and Titgemeyer, 2007a). In dairy calves, reticulorumen pH seems to be consistently lower than in adult cattle, ranging from 5.0 to 6.0 (Gentile et al., 2004, Suarez-Mena et al., 2016, Yohe et al., 2018). Despite presenting lower reticulorumen pH compared to adult cows, calves are still prone to decreases in reticulorumen pH, especially during weaning as they transition from liquid to solid diets (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020). To our knowledge, no clear SARA thresholds have been defined for calves.

Subacute reticulorumen acidosis has been defined as a digestive disorder in dairy cows characterized when reticulorumen drops pH between 5.5 and 5.0 (Garrett et al., 1999a). This SARA threshold was highly based on work by Russell and Dombrowski (1980) showing that reticulorumen bacterial growth seems to be compromised at lower pH. Other authors argument that the threshold to define SARA should be raised to 5.8 instead of 5.5, as at this pH fiber digestion is negatively affected (Krause et al., 2002, Sung et al., 2006). More recently, indwelling reticulorumen pH measuring systems have been used to constantly monitor reticulorumen pH dynamics in cattle (Penner et al., 2009). Gozho et al. (2007) utilized indwelling reticulorumen pH monitoring systems and constant reticulorumen fluid sampling to investigate the relationship between reticulorumen pH and free ruminal lipopolysaccharides (LPS). The authors suggested that SARA should be defined as combination of time and pH as cows displayed increases in blood acute phase proteins and reticulorumen endotoxins when they spent more than 180 minutes per day

under pH 5.6 (Gozho et al., 2007). Both SARA definition pH thresholds (< 5.8 and < 5.6) have been vastly adopted by researchers, and it is common practice to analyze and report data utilizing both thresholds (Kleen et al., 2013, Danscher et al., 2015).

Subacute reticulorumen acidosis is difficult to detect on farm as animals might not display clinical symptoms. Furthermore, single point measurements of reticulorumen pH do not always reflect pH variations an animal experiences throughout the day. Hence, it is important to understand the causes of the disorder so its risk can be minimized through management strategies. Thus, in the next section we will explore the causes of SARA.

1.3.2 *Causes of SARA*

Subacute reticulorumen acidosis is caused by one or a combination of two factors: i) replacement of fiber by non-fibrous carbohydrates to meet elevated energy requirements; ii) lack of reticulorumen adaptation (Kleen et al., 2003, Plaizier et al., 2018, Plaizier et al., 2022). Diets rich in non-fibrous carbohydrates are associated with decreases in reticulorumen pH and diets rich in fiber are associated with increases in reticulorumen pH. In ruminants, high starch diets have been shown to favor the production of volatile fatty acids (VFA) and lactate in the reticulorumen (Nafikov and Beitz, 2007). This can be attributed to the observed increases in amylolytic bacteria populations, especially the acid-tolerant and lactate-producing *Streptococcus bovis* (Russell, 2002, Wang et al., 2015). Lactate is a stronger acid than VFA, thus lactate accumulation is associated rapid pH declines in the reticulorumen (Dijkstra et al., 2012). Drastic drops in reticulorumen pH have been observed in calves fed diets with elevated proportions of non-fibrous carbohydrates. These decreases in calf reticulorumen pH happen especially during weaning

as calves transition from liquid to solid diets (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020).

Cows are fed mostly forage based diets during the dry period, (Kleen et al., 2003). Consequently, reticulorumen VFA concentration is lower during the dry period, which has a negative impact on reticulorumen papillae size (Dieho et al., 2017). After parturition, the exposure to elevated VFA concentrations promotes reticulorumen papillae development (Steele et al., 2015, Dieho et al., 2017). However, authors reported that papillae growth during the postpartum period is not immediately apparent, which might affect VFA absorption and culminate in excessive accumulation, and, consequently, reticulorumen pH drops (Kleen et al., 2003, Dieho et al., 2017). Greater papillae dimensions have been associated with greater VFA absorption and possible reductions in SARA. Furthermore, Nishihara et al. (2023) reported a positive association between reticulorumen papillae dimensions and genes associated with VFA absorption and ketogenic activity. Thus, avoiding abrupt transitions to diets containing low fiber and non-fibrous carbohydrates is recommended (Humer et al., 2018).

Subacute reticulorumen acidosis is caused by a combination of abrupt increases in dietary non-fibrous carbohydrates and lack of reticulorumen preparation, which is commonly observed in during the weaning and transition periods for calves and cows, respectively. Thus, in the next section we will explore the literature investigating the effects of SARA on reticulorumen microbiome, fermentation end products, and epithelial health in cows and calves.

1.3.3 *Effects of SARA on reticulorumen microbiome, VFA production, and epithelial health*

Low reticulorumen pH for prolonged periods of time has been associated with changes in the reticulorumen microbiome and VFA concentrations. These drastic changes have been associated with poor feed digestion, damage to the reticulorumen epithelium, and overall poor performance.

The research on the effects of SARA on the reticulorumen microbiome and fermentation end products in calves is still limited. However, the available research seems to indicate that the reticulorumen microbiome in calves responds to SARA in a similar way that adult cows. For example, Watanabe et al. (2019) reported that calves also displayed decreases in bacterial diversity, decreases in acetate, and increases in propionate concentration when SARA was induced. In addition, Gelsinger et al. (2020) reported that calves with calf starter induced SARA had greater concentrations of total VFA, as well as greater concentrations of propionate and isobutyrate compared to calves fed a control calf starter.

Decreases in dairy cow reticulorumen microbial richness and diversity under low reticulorumen pH has been reported by several researchers (Khafipour et al., 2016, Plaizier et al., 2017b). Zhou et al. (2015) reported that cellulolytic bacteria are the most susceptible to SARA as their optimal pH range for growth is between 6 and 7. Decreases in cellulolytic bacteria abundance (e.g. *Fibrobacter succinogenes* and *Ruminococcus albus*) have also been reported in Holstein steers under a SARA inducing diet compared to control (Ogunade et al., 2019). Consequently, fiber digestion is impaired under low reticulorumen pH. In fact, Russell et al. (2009) reported that the passage of undigested cellulose from the reticulorumen to the other forestomachs increases as reticulorumen pH decreases.

Furthermore, an in-vitro study investigating the relationship between reticulorumen pH on bacteria attachment to substrate and fiber digestion, Sung et al. (2006) reported that the number of *Fibrobacter succinogenes, Ruminococcus flavefaciens,* and *Ruminococcus albus* attached to substrate at pH 5.7 were lower compared to pH 6.2 and 6.7. In addition, the authors reported that dry matter digestibility and total VFA production was lower at pH 5.7 compared to pH 6.2 and 6.7 (Sung et al., 2006). Changes in VFA have also been observed in-vivo, Plaizier et al. (2017a) reported that cows under grain-induced SARA displayed reduced acetate to propionate ratio. These changes in acetate to propionate ratio might be explained by the increased *Prevotella sp.*, S*elenomonas. ruminantium*, and *Megasphaera elsdenii* populations observed in dairy cows under experimentally-induced SARA (Plaizier et al., 2017b).

Low pH for prolonged periods of time has been associated with damage to the reticulorumen epithelium in calves and cows. Studies have reported increased reticulorumen LPS concentrations in calves induced SARA (Gozho et al., 2007, Watanabe et al., 2019). Furthermore, Li et al. (2019) reported that calf starter induced SARA affected biological pathways associated with cell division, possibly affecting reticulorumen epithelial health and anatomical development in calves. In addition, calves fed a SARA-inducing calf starter [Calf Starter Information: Pelleted, 42.7% starch, 15.1% neutral detergent fiber, 57.8% non-fibrous carbohydrates] tended to have a lesser reticulorumen weight and displayed greater reticulorumen lesion scores compared with calves fed a calf starter designed to blunt SARA [Calf Starter Information: texturized, 35.3% starch, 25.3% neutral detergent fiber, 48.1% non-fibrous carbohydrates]; Gelsinger et al., 2020). Lastly, Meissner et al. (2017) reported that short exposure to reticulorumen pH and VFA

concentrations similar to the ones observed in calves were sufficient to damage tight junction proteins and disturb epithelial barrier function in sheep.

Increased reticulorumen LPS concentrations have also been observed in cows under induced SARA (Gozho et al., 2007). Furthermore, elevated concentrations of reticulorumen LPS in cows experiencing SARA have been associated with local (Zhao et al., 2018) and systemic (Gozho et al., 2007) inflammation responses. Furthermore, in mature cows under induced SARA, Steele et al. (2011) reported reductions in the depth of the stratum basale, spinosum, and granulosum layers of the reticulorumen epithelium.

Damages to the reticulorumen epithelium have been associated with the incidence of liver abscesses, as they might allow microorganisms to enter the bloodstream (Nagaraja and Titgemeyer, 2007a, Elmhadi et al., 2022). Lastly, long exposure to SARA can lead to metabolic acidosis, which has been associated with lameness in adult cows and death in calves (Gentile et al., 2004, Passos et al., 2023). These negative effects of SARA on reticulorumen environment and health might affect animal performance and behavior. Thus, in the next section of this review, we will explore the literature investigating the effects of SARA on the performance and behavior of calves and cows.

1.3.4 *Effects of SARA on animal performance and behavior*

Subacute reticulorumen acidosis can affect the reticulorumen microbiome and fermentation dynamics, which might affect animal performance and behavior. Gelsinger et al. (2020) reported that calves with induced SARA had decreased solid feed dry matter intake (DMI) and body weights compared to control. Likewise, Li et al. (2019) reported a

significant impact of induced SARA on solid feed DMI and body weight in calves starting at four and five weeks of age, respectively.

Luan et al. (2016) reported that lactating cows under induced SARA displayed reduced feed intake, reduced milk yield, and tended to display reduced milk fat yield. More recently, in a survey of 261 cows from 32 farms across 3 regions (Australia, California, and Canada), Golder et al. (2023) utilized a combination of reticulorumen fluid pH, ammonia, lactate, and VFA concentrations to perform a cluster analysis and classify cows as being under high or low SARA risk, reporting no differences in milk yield between SARA risk groups. However, the authors reported that cows under high SARA risk displayed lower milk fat percentage (Golder et al., 2023). It is believed that the low reticulorumen pH observed might affect the biohydrogenation of unsaturated fats in the reticulorumen, increasing the concentration of *trans*-10 *cis*-12 conjugated linoleic acid, which can inhibit milk fat synthesis in the mammary gland (Plaizier et al., 2018).

It is believed that the reduced dry matter intake displayed by cows and calves under SARA is associated with one or a combination of factors: i) reticulorumen distention and gut fill caused by reduced fiber digestibility; ii) stimulation of reticulorumen epithelial receptors by the increased VFA concentration; iii) increased flow of propionate to the liver (Allen, 2000, Allen et al., 2009).

Subacute reticulorumen acidosis has also been associated with changes in behavior in dairy cows. For example, DeVries et al. (2008) reported that early lactation cows fed a 45% forage diet were under high SARA risk and sorted in favor of long particles and against short particles in the diet (DeVries et al., 2008). Similarly, in a study evaluating sorting in early lactation cows, Coon et al. (2019) reported that cows under high SARA

risk displayed feed sorting behaviors for long particles. To our knowledge, there were no studies evaluating the effects of SARA on the feeding behavior of calves. However, in a study with Angus heifers under induced SARA, DeVries et al. (2014) reported that heifers sorted for long particles and against fine particles in the diet. Furthermore, the authors reported a positive association between time under reticulorumen pH 5.5 and greater sorting for long particles in the diet (DeVries et al., 2014). Nutritional disorders such as SARA have been associated with the display of non-nutritive oral behaviors in ungulates (Bergeron et al., 2006). Non-nutritive behaviors such as tongue rolling, excessive licking, and oral manipulation are normally displayed by calves (Horvath and Miller-Cushon, 2017). Still, the excessive display of these non-nutritive oral behaviors has been associated with poor animal welfare (Mason and Latham, 2004).

Subacute reticulorumen acidosis can affect the reticulorumen environment of calves and cows. In addition, both cows and calves seem to have their performance and feed intake affected by SARA. Subacute reticulorumen acidosis might be difficult to detect using a single variable in the field, and the symptoms might be confounded with other disorders or vary in expression according to each animal. Consequently, most research work focuses on the prevention of the disorder as treatment might be labor intensive and mostly recommended for severe acute reticulorumen acidosis cases (Snyder and Credille, 2017). Thus, in the next section of this review, we will explore the literature investigating the nutritional strategies that can be utilized to prevent SARA in calves and cows.

1.3.5 *Nutritional strategies to prevent SARA*

Subacute reticulorumen acidosis might be caused by one or a combination of two factors: i) replacement of fiber by non-fibrous carbohydrates to meet elevated energy

requirements; ii) lack of reticulorumen adaptation (Kleen et al., 2003, Plaizier et al., 2018, Plaizier et al., 2022). Thus, the most common SARA prevention strategies tend to target one of the main causes of SARA. Hence the following section will focus on common nutritional strategies that can be adopted to prevent SARA.

1.3.5.1 Preventing SARA with forage

One of the most common strategies is to adjust the forage content of the diet. In fact, forage NDF (fNDF) has greater positive effect on reticulorumen pH than total dietary NDF (Allen, 1997). The NASEM (2021) recommends that, for optimum reticulorumen pH, dietary fNDF should be between 17 and 27% of diet DM. In addition, Yoder et al. (2013) reported that feeding diets ranging from 21.5 to 28% fNDF was associated with increases in odd and branched-chain VFA that are synthetized mainly by cellulolytic bacteria (e.g. iso-C14:0, iso-C15:0, and iso-C16:0). Another fiber management strategy utilized for SARA prevention is the management of the percentage of physically effective NDF (peNDF) in the diet. The concept of peNDF integrates feed particle size (between 1.88 and 8 mm) and NDF to estimate the time animals spend chewing feed and ruminating (Mertens, 1997, Zebeli et al., 2012). Indeed, a recent meta-analysis Souza et al. (2022) reported a positive linear relationship between rumination time per day and reticulorumen pH. Furthermore, the positive association between peNDF inclusion and reticulorumen pH in lactating dairy cows has been highlighted in a meta-analysis evaluating 33 studies and 135 diets (Khorrami et al., 2021). The meta-analysis authors suggest that diets should contain between 15 and 18% peNDF (> 8.0 mm) to prevent SARA in cows fed diets containing between 35 and 40 % non-fibrous carbohydrates (Khorrami et al., 2021). In calves, a trial mixing straw with calf starter pellets to increase particle size in the diet,

reported that calves fed calf starter and straw chopped at 7.10 mm had greater reticulorumen pH and similar performance and reticulorumen development compared to calves fed calf starter and straw chopped at 3.04 mm (Suarez-Mena et al., 2016).

Cows under SARA risk tends to perform sorting behaviors towards long feed particles (DeVries et al., 2014). Therefore, a valid strategy to prevent SARA occurrence in cows and calves is to provide a free-choice forage source. Keunen et al. (2003) reported that cows offered free choice hay during induced SARA showed an increase in free-choice hay intake. Furthermore, the authors reported that cows undergoing induced SARA with free access to hay had similar milk yield and similar time under reticulorumen pH 5.6 compared to control (Keunen et al., 2003). Similarly, Monjezi et al. (2022) reported that lambs offered free access to wheat straw did not display decreases in dry matter intake or average daily gain, despite being fed diets with different starch levels.

Nutritional strategies based on dietary fiber management rely on promoting rumination to regulate pH via saliva buffers (Plaizier et al., 2018). Another SARA prevention strategy consists in supplementing diets with feed additives such as exogenous buffers and antimicrobials. Thus, the section below will explore the effects of dietary inclusion of exogenous buffers and antimicrobials on SARA.

1.3.5.2 Preventing SARA with feed additives

Buffers such as sodium bicarbonate or alkalinizing agents such as magnesium oxide are commonly added to diets with the objective of stabilizing reticulorumen pH (Plaizier et al., 2018). In a study investigating the efficacy of sodium bicarbonate (0.8 % inclusion rate) and a magnesium oxide mix (0.4 % inclusion rate) in dairy cows under induced SARA, Bach et al. (2018) reported that both exogenous buffers were able to prevent drops

in reticulorumen pH and milk yield. Recently, Guo et al. (2023) mixed a liquid alkaline mineral buffer complex to transition cow diets and observed increased reticulorumen microbiota richness, increased reticulorumen pH, and increased milk yields in supplemented cows compared to control. On the other hand, Lobo et al. (2023) reported that dietary buffer inclusion improved overall total tract dry matter and NDF digestibility but did not affect reticulorumen pH or fermentation metabolites in lactating cows.

Providing animals with free-choice buffers might also be a valid strategy to prevent SARA. However, palatability of free-choice buffers might be an issue as Keunen et al. (2003) reported elevated individual variation regarding buffer intake and no effects of providing cows with a free-choice buffer on reticulorumen pH or SARA length. On the other hand, cows under induced SARA with free access to a molasses and sodium bicarbonate block showed a lesser reticulorumen pH decrease and lesser SARA length and intensity compared control. Lastly, Paton et al. (2006) reported that cows with free access to sodium bicarbonate had similar reticulorumen pH, SARA length and intensity to cows supplemented (0.7 % inclusion) with sodium in the diet. However, the authors reported that mixing sodium bicarbonate in the diet reduced the number of SARA bouts compared to offering free-choice access to the buffer (Paton et al., 2006).

Another possible SARA prevention strategy consists in the addition of antimicrobials such as virginiamycin and monensin focuses on managing the reticulorumen microbiome mostly by selecting against pathogenic or lactate producing bacteria (Plaizier et al., 2018). For example, Coe et al. (1999) reported that Holstein steers displayed lower reticulorumen counts of *Streptococcus bovis*, *Lactobacillus*, and *Fusobacterium necrophorum* when supplemented with virginiamycin (175 or 250

mg/steer/day) compared to control. Furthermore, steers supplemented with virginiamycin showed increased reticulorumen pH and decreased reticulorumen lactate after induced SARA (Coe et al., 1999).

Dietary supplementation with ionophores, especially monensin, has been associated with inhibition of reticulorumen gram-positive bacteria, especially lactate producers, without affecting lactate-utilizing bacteria (Weimer et al., 2008). Mutsvangwa et al. (2002) reported that dietary supplementation with monensin (22 mg/kg) was associated with increases in dry matter intake and milk yield in dairy cows under induced SARA. However, the authors did not observe any effects of monensin inclusion on reticulorumen pH or VFA concentration (Mutsvangwa et al., 2002). On the other hand, Pacheco et al. (2023) did not report effects of monensin supplementation (3g/cow/day) on dry matter intake or milk yield but reported increases in reticulorumen pH and propionate concentration compared to control. In dairy calves, antimicrobial supplements have been mostly associated with promoting lower gut health and as a prevention for diarrhea. In dairy calves, Salazar et al. (2019) reported that supplementation with monensin (30 mg/g of calf starter) was not associated with increases in intake, weight gain, or feed efficiency. However, calves supplemented with monensin had lower fecal *Escherichia coli* counts and greater fecal consistency when compared to control (Salazar et al., 2019).

Although dietary supplementation with antimicrobials seems to be effective, there are plenty of efforts to reduce antimicrobial usage in food animal production, especially in Europe (More, 2020). With the prohibition of monensin as a feed additive for cattle in Europe (EuropeanCommision, 2007), researchers started investigating natural alternatives such as probiotics to improve animal health and performance (Cangiano et al., 2020,

Barreto et al., 2021). Hence, in the next section of this review we will first define what probiotics are and explore the literature regarding the use of probiotics in cows and calves under SARA risk.

1.4 Probiotics and SARA

Probiotics provide a natural alternative to the use of dietary antimicrobials for SARA prevention. Probiotics are defined as live strains of select microorganisms which might grant a health benefit to the host (Markowiak and Śliżewska, 2017). When developing a probiotic it is essential that the strains chosen are safe for human and animals, specific for a target disorder, and that they can survive in the gastrointestinal tract (Anadón et al., 2006). The use of probiotics as a SARA prevention tool is based mainly on their potential to modulate reticulorumen fermentation and microbiome either by stimulating lactate-utilizing bacteria growth (Callaway and Martin, 1997), increasing lactate utilization (Henning et al., 2010b), oxygen consumption (Marden et al., 2008), competitive exclusion for substrates (Counotte et al., 1981b, Callaway et al., 2008), or production of bacteriocins (Hegarty et al., 2016). For example, (Yoon and Stern, 1996) supplemented lactating dairy cows with either a live fungal (*Aspergillus oryzae*, 3 g/d) or yeast (*Saccharomyces cerevisiae*, 57 g/d) cultures and reported that supplementing cows with the fungal culture was associated with increases in proteolytic and cellulolytic bacteria counts. In addition, the authors reported that feeding the live yeast culture promoted reticulorumen proteolytic bacteria growth (Yoon and Stern, 1996). In another study, Marden et al. (2008) fed a live yeast strain (*Saccharomyces cerevisiae*, 5g/d) to high-yielding lactating cows fed diets containing high NFC and reported that cows fed the live yeast strain displayed less reticulorumen lactate and greater reticulorumen pH, NDF

digestibility, acetate , propionate, and total VFA concentration compared to control. Bach et al. (2007) reported similar effects of live yeast supplementation (*Saccharomyces cerevisiae*, 5g/d, 10^{10} CFU/d) on reticulorumen pH of cows fed in robotic milking systems. Furthermore, the authors also reported that cows supplemented with live yeast displayed increased meal frequency compared to control (Bach et al., 2007). Goto et al. (2016) fed a probiotic containing a blend of lactate producing (*Lactiplantibacillus plantarum*), lactate-utilizing (*Enterococcus faecium*), and butyrate-producing (*Clostridium butyricum*) bacteria to dairy cows fed SARA inducing diets and reported decreases in reticulorumen lactate and increases in reticulorumen pH and dry matter intake associated with probiotic supplementation.

In Holstein calves provided exclusively calf starter, Qadis et al. (2014) reported that supplementation of a bacteria-based probiotic (*Lactiplantibacillus plantarum*, *Enterococcus faecium*, and *Clostridium butyricum*, 1.5g or 3g/100kg of body weight/day) was associated with decreased reticulorumen lactate and increased reticulorumen pH. Furthermore, in a study supplementing a live yeast (*Saccharomyces cerevisiae*, 1.5 x 10^6 CFU/g) to Holstein calves fed calf starter without access to forage, Terré et al. (2015) reported that calves receiving the yeast probiotic tended to display greater solid feed intake and greater average daily gain during the preweaning period. Furthermore the authors reported that calves supplemented with the yeast probiotic tended to display greater average daily gain and displayed increased solid feed intake, reticulorumen pH, and reticulorumen populations of *Ruminococcus albus* during the postweaning period compared to control (Terré et al., 2015). Lastly, in recent reviews Alawneh et al. (2020) and Cangiano et al. (2020) reported that a most studies evaluating the use of probiotics in

calves focuses mainly on lower gut health and diarrhea prevention and generally yield positive effects on calf health and performance, especially when animals are subject to stress.

Overall, cows and calves seem to benefit from probiotic supplementation. However, choice of probiotic microorganism must be specific for the challenge you're trying to alleviate. In the case of SARA, supplementing cows with lactate utilizing bacteria seems reasonable as they metabolize lactate into VFA with lesser acidifying potential. *Megasphaera elsdenii* is the main reticulorumen bacteria responsible for metabolizing lactate into VFA but its adoption as a probiotic still seems to be limited (Plaizier et al., 2018). Thus, the next section of this literature review will further describe *Megasphaera elsdenii* importance of in reticulorumen pH regulation and its potential as a probiotic to prevent SARA. Lastly, we will describe the current literature regarding the utilization of probiotics co *Megasphaera elsdenii* in calves and cows under SARA risk.

1.4.1 *Megasphaera elsdenii*

Megasphaera elsdenii is a gram-negative, lactate-utilizing, and naturally occurring reticulorumen bacteria. In fact, its presence has been detected in the reticulorumen of calves in the first day of life (Jami et al., 2013). In adult cattle, fed forage based diets, Klieve et al. (2003) reported *Megasphaera elsdenii* be 10^4 CE/ml. The same authors reported that *Megasphaera elsdenii* populations displayed drastic increases 24h upon increasing the non-fibrous carbohydrate inclusion to the diets, also displaying greater population increase than the lactate-producer *Streptococcus bovis* (Klieve et al., 2003). *Megasphaera elsdenii* preferred substrates are lactate, maltodextrins, and amino acids and

its products are propionate, acetate, butyrate, and branched chain VFA (Russell, 2002). Lactate is a stronger acidifying agent compared to all VFA, thus its accumulation is associated with rapid pH declines in the reticulorumen (Dijkstra et al., 2012). However, *Megasphaera elsdenii* is able to prevent lactate accumulation by metabolizing it to less acidifying VFA. Furthermore, Nishihara et al. (2023) reported that increased VFA concentrations were associated with increased expression in genes associated with VFA absorption and ketogenic activity in the reticulorumen epithelium. Therefore by metabolizing lactate and preventing its accumulation, *Megasphaera elsdenii* plays a major role in reticulorumen pH stabilization (Dijkstra et al., 2012, Wang et al., 2015). In fact, Counotte et al. (1981a) reported that *Megasphaera elsdenii* can successfully compete for substrate against other lactate utilizing bacteria, making it responsible for 60 to 80% of the lactate utilization under normal reticulorumen conditions. However, *Megasphaera elsdenii* seems to also be extremely important for reticulorumen pH stabilization under SARA conditions. Chen et al. (2019) reported that *Megasphaera elsdenii* was able to demonstrate populational growth and utilized lactate at a faster rate than it was being produced in an *in vitro* study mimicking prolonged SARA conditions. In another *in vitro* study, Meissner et al. (2014) reported that *Megasphaera elsdenii* did not have its reticulorumen activity impacted by the presence by antimicrobials commonly utilized in dairy cattle production. In fact, the authors reported that the interaction between *Megasphaera elsdenii* and the antimicrobials was additive.

Megasphaera elsdenii has the potential to be used as tool to prevent SARA in cattle as it is naturally occurring, able to persist and function under low pH, and does not seem to be affected by some common antimicrobials. Consequently, *Megasphaera elsdenii*

strains have been patented and are now used as SARA control tools (Leedle et al., 1990; Horn et al., 2009). The rationale behind utilizing *Megasphaera elsdenii* as a SARA prevention tool consists of introducing lactate-utilizing bacteria into the rumen to increase existing populations of the bacteria until they can proliferate on their own. In fact, Klieve et al. (2003) demonstrated that *Megasphaera elsdenii* populations were able to persist and increase in size after being inoculated in the reticulorumen of beef steers. However *Megasphaera elsdenii* adoption as SARA prevention tool in the field still seems to be limited (Plaizier et al., 2018). In a recent meta-analysis, Susanto et al. (2023) showed that the majority of the current literature on the use of *Megasphaera elsdenii* as a SARA prevention tool were conducted in beef cattle or sheep. Briefly, the authors reported that *Megasphaera elsdenii* supplementation in beef and sheep were associated with increased average daily gain, hot carcass weights, and decreases in liver abscess occurrence (Susanto et al., 2023). Researchers have investigated the effects of *Megasphaera elsdenii* supplementation as a SARA prevention tool in calves and cows, but the literature is still limited. The next section will describe the current literature regarding the use of *Megasphaera elsdenii* probiotic supplementation as a SARA prevention tool in calves and cows.

1.4.1.1 Megasphaera elsdenii as a SARA prevention tool in cows and calves

The current literature on *Megasphaera elsdenii* supplementation in calves is still limited and has yielded conflicting results. In a study supplementing calves with an oral dose of *Megasphaera elsdenii* at 14 days of age, Muya et al. (2015) reported that calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age displayed greater dry matter intake, greater weaning weight, and tended to have greater average daily gain

compared to control calves. In addition, the authors reported that calves receiving an oral dose of *Megasphaera elsdenii* probiotic at 14 days of age had wider reticulorumen papillae than control calves (Muya et al., 2015). In a study investigating the effects of a single *Megasphaera elsdenii* dose in calves at 14 days of age fed *ad-libitum* milk, Muya et al. (2017) reported that calves receiving the *Megasphaera elsdenii* supplementation were heavier at weaning and showed greater solid feed dry matter during the preweaning and postweaning periods compared to control. Lastly, Yohe et al. (2018) reported that providing calves with *Megasphaera elsdenii* oral supplementation at 14 days of age did not seem to affect body weight or average daily gain.

Research utilizing *Megasphaera elsdenii* supplementation as a SARA prevention tool is still limited and sometimes yields conflicting results. For example, Aikman et al. (2011) reported that cows receiving *Megasphaera elsdenii* supplementation via drench immediately after calving spent less time with a reticulorumen pH < 5.6 and had similar yields to cows drenched with a placebo. In a study evaluating the effects of *Megasphaera elsdenii* supplementation via oral drench in periparturient cows, Stevens et al. (2017) administered a drench containing with the probiotic two weeks before the expected calving date, within 3 days post calving, or both pre and post calving. The authors reported that cows with 3 or more lactations dosed with the probiotic prepartum displayed greater milk yield compared to control and the other probiotic treatments. In addition, the authors reported a numerical decrease in subclinical ketosis in cows that received *Megasphaera elsdenii* supplementation via oral drench pre- and post-partum (Stevens et al., 2017)

The current literature on *Megasphaera elsdenii* supplementation to calves and cows under SARA risk is still limited and shows either positive or inconclusive results. Thus,

there is an opportunity to further investigate the applicability of *Megasphaera elsdenii* supplementation as a SARA prevention tool and its effects on the production, behavior, and reticulorumen environment of dairy calves and cows.

1.5 CONCLUSION

Diets rich in non-fibrous carbohydrates favor the production of VFA in the reticulorumen that is used by cows as an energy source. However, these diets also favor the production of lactate in the reticulorumen. The accumulation of lactate and VFA in the reticulorumen causes reticulorumen pH to decrease and increases the risks of the animal experiencing SARA. The effects of SARA on dairy cows have been deeply studied. In fact, studies have reported that SARA can damage the reticulorumen epithelium, affect feeding behavior, and cause poor performance in cows. Recently, researchers have proposed that calves might also experience SARA. In fact, studies have shown that calves also experience decreases in reticulorumen pH. These reductions in reticulorumen pH are mostly observed around weaning as calves are transitioning from liquid to solid diets. There is limited research on the effects of SARA in calves, studies have reported that it might be associated with poor performance and reticulorumen development. Therefore, there is an opportunity to further investigate how SARA affects the reticulorumen environment, performance, behavior, and health of calves.

Animals experiencing SARA are mostly asymptomatic, which makes it difficult to be diagnosed in the field without a multifactorial approach. Hence, most effort is put towards its prevention instead of treatment. There are many SARA prevention strategies that can be adopted on-farm ranging from dietary adjustments to the use of antimicrobials. One of the many effective SARA prevention strategies consists in supplementing animals

with probiotics containing lactate-utilizing bacteria such as *Megasphaera elsdenii*. In fact, *Megasphaera elsdenii* plays a key role in reticulorumen pH regulation as it is responsible for fermenting most of the reticulorumen lactate. Furthermore, *Megasphaera elsdenii* can persist and function under low pH and does not seem to be inhibited by common antimicrobials utilized in dairy cattle production. Hence, *Megasphaera elsdenii* has the potential to be used as tool to prevent SARA in cows and calves. The limited research available on the use of *Megasphaera elsdenii* supplementation as a SARA prevention strategy in cows and calves has yielded positive results. However, adoption of *Megasphaera elsdenii* supplementation as SARA prevention tool in the field is still limited. Therefore, there is an is an opportunity to develop applicable and effective SARA prevention strategies utilizing probiotics containing *Megasphaera elsdenii*.

1.6 DISSERTATION OBJECTIVES

The overall objective of this dissertation is to develop practical *Megasphaera elsdenii* supplementation strategies to prevent SARA therefore improving welfare and performance in cows and calves. The objective of Chapter 2 of this dissertation is to investigate the effects of two different *Megasphaera elsdenii* supplementation strategies on reticulorumen pH, milk yield, feed intake, and feeding behavior of lactating cows undergoing a grain-induced SARA challenge. As the current literature investigating the effects and prevention strategies of SARA in calves is extremely limited, Chapters 3 and 4 will focus on investigating the effects of strategic *Megasphaera elsdenii* supplementation in calves. More specifically, Chapter 3 of this dissertation aims to evaluate the effects of two different strategic interventions *Megasphaera elsdenii* supplementation via oral capsule on feed intake, performance, and feeding behavior patterns of dairy-beef crossbred

calves. To further consider the effects of strategic probiotic interventions proposed in Chapter 3, Chapter 4 of this dissertation aims to evaluate the effects of two different strategic interventions with *Megasphaera elsdenii* supplementation via oral capsule on reticulorumen pH, VFA, and anatomical development of dairy beef-crossbred calves. Lastly, Chapter 5 of this dissertation will present a summary of the main findings from Chapters 2 through 4 and discuss the implications of these results.

CHAPTER 2. EFFECTS OF A *MEGASPHAERA ELSDENII* ORAL DRENCH ON RETICULORUMEN PH DYNAMICS IN LACTATING COWS UNDER SUBACUTE RUMINAL ACIDOSIS CHALLENGE

2.1 INTRODUCTION

Subacute ruminal acidosis (SARA) is a disorder characterized by extended periods of ruminal pH below 5.6 (Garrett et al., 1999b, Gozho et al., 2005, Plaizier et al., 2008). In grain-adapted cattle, normal rumen pH ranges from 5.5 to 6.5 (Nagaraja and Titgemeyer, 2007b). However, cows fed diets rich in grains and non-fibrous carbohydrates have an increased risk of experiencing SARA (Krause and Oetzel, 2006b). Around 19 % of early lactation and 26 % of mid-lactation dairy cows experience SARA (Garrett et al., 1997). The systemic impact of acidosis can have severe implications such as laminitis, which is highly associated with lameness (Nocek, 1997). Lameness is one of the most important causes of premature involuntary culling (Krause and Oetzel, 2006b).

Megasphaera elsdenii is a gram-negative rumen microorganism known for metabolizing lactic acid and helping to stabilize ruminal pH (Counotte et al., 1981a, Nocek, 1997, Nagaraja and Titgemeyer, 2007b). It can ferment lactic acid to acetic and propionic acids (Hino et al., 1994). Some strains of *Megasphaera elsdenii* have been patented and are now used as acidosis control tools (Leedle et al., 1990, Horn et al., Inventors. 2009). The prevention strategy consists of introducing lactate-utilizing bacteria into the rumen to increase existing populations of the bacteria until they are able to proliferate on their own. Klieve et al. (2003) demonstrated that the use of *Megasphaera elsdenii* allowed bacterial populations to establish 5 to 7 d earlier in inoculated animals when compared to non-inoculated animals. One study found that high yielding (> 10,000 kg milk/lactation)

lactating dairy cows fed a high-starch diet and drenched with *Megasphaera elsdenii* had increased milk yield compared to non-drenched cows (Aikman et al., 2009), but this was not supported in a later study by Aikman et al. (2011), where early lactation cows drenched with *Megasphaera elsdenii* had similar yields to cows drenched with a placebo. However, in the study by Aikman et al. (2011), cows drenched with *Megasphaera elsdenii* spent less time with a rumen pH < 5.6 compared to cows drenched with a placebo.

Drenching cows with *Megasphaera elsdenii* may offer benefits to rumen health and production. However, there is a lack of evidence on how this tool improves rumen pH, and its effect on feeding behavior and milk production in cows under increased risk of SARA. The objective of this study was to evaluate the effects of a *Megasphaera elsdenii* oral drench on reticulorumen pH, milk yield, and milk components (fat and protein), as well as feed intake, feeding time and the number of feeder visits of lactating cows undergoing a ruminal acidosis challenge. We hypothesized that cows drenched with *Megasphaera elsdenii* would have a better ruminal environment, which could possibly increase the number of feeder visits and feeding time, consequently increasing DMI and performance.

2.2 MATERIALS AND METHODS

2.2.1 *Animal Housing and Diet*

The experiment was conducted at the University of Kentucky Coldstream Dairy Research Farm in Lexington, KY, between March and August 2017, under Institutional Animal Care and Use Committee protocol number 2017-2585. A total of 16 mid-lactation Holstein dairy cows (1.4 ± 0.9 lactations) averaging 670.0 ± 87.0 kg body weight and producing 42.1 ± 10.3 kg/d of milk were enrolled in this study.

The experimental pen comprised a compost bedded pack equipped with 8 automatic intake recording feeders (Insentec, Hokofarm Group, Marknesse, the Netherlands). These feeders precisely measure feed intake and behaviors such as number of visits to the feeder and time spent feeding as validated by Chapinal et al. (2007). We performed two cross-over trials with 8 cows each. Each cow had a reticulorumen bolus that recorded reticulorumen pH in 10-min intervals (iNVOTEC Animal Care, smaXtec Animal Care, Graz, Austria). The bolus measures 132 x 35 mm and has previously been validated in rumen-cannulated dairy cows (Klevenhusen et al., 2014). One week prior to the start of the experimental period, boluses were individually calibrated utilizing a pH 7.0 buffer solution and orally administered to the cows utilizing a bolus gun.

Cows were fed a TMR formulated following the National Research Council (NRC) guidelines (NRC, 2001) to meet or exceed the requirements of lactating dairy cows producing at least 39 kg of milk daily. Diet nutrient compositions for each trial are shown in Table 2.1. Cows were fed *ad libitum* twice per day at 0800 and 1430 h. Orts were removed once per day before the 1430 h feeding. Animals had *ad libitum* access to fresh water provided from a self-filling water trough located in the feeding alley. Milking occurred twice daily at 0700 and 1600 h. Daily milk yield was recorded using an automatic meter (AfiMilk, AfiMilk, Kibbutz Afikim, Israel). Milk fat and protein were measured twice daily using an in-line milk analyzer (AfiLab, AfiMilk, Kibbutz Afikim, Israel) validated by (Kaniyamattam and De Vries, 2014).

2.2.2 *Experimental Design*

This study consisted of two crossover trials to determine the effectiveness of a drench containing a live culture of *Megasphaera elsdenii* NCIMB 41125 (Lactipro Advance®, MS Biotec, Wamego, KS). The two trials were identical except for the administration time of the live culture of *Megasphaera elsdenii* which occurred at day 4 and day 1 prior to the acidosis challenge for trial 1 and 2, respectively. Each crossover consisted of two eight-day experimental periods separated by a 4 wk washout period. Each trial day was defined by the 24 h interval between the afternoon feedings. Therefore, study days started and ended at 1430 h. Cows were randomly assigned to one of two trial groups (Trial 1; n = 8; Trial 2; n = 8) and moved to the experimental pen 1 wk before the start of the study for habituation with the automatic feeders, pen mates, and the experimental pen. Each animal was assigned to one individual feeder using each cow's radio frequency identification (RFID) tag to operate and record the feed intake. Each animal ate from their assigned bin until the end of the experimental period.

The first 3 d of each experimental period (d_{-4}, d_{-3}, d_{-2}) were considered baseline days. On the fourth day of the experimental period (d_{-1}) cows had their feed allowance reduced by 50%, based upon individual average dry matter intake during the baseline period. On the fifth day of the experimental period (d_0), cows received a challenge mix with elevated proportions of non-fibrous carbohydrates to induce SARA. The challenge mix consisted of 2 kg of rolled barley, 2 kg of ground wheat and 0.9 kg of molasses that was combined with 4.3 kg of TMR and offered to the animals for 2 h (Table 2.1). Orts were weighed and subsequently replaced with TMR offered *ad libitum*. The last 3 d of each experimental period (d_{+1}, d_{+2}, d_{+3}) were considered recovery days. Milk yield and

components, dry matter intake (DMI) and feeding behavior (time spent feeding and number of visits to the feeder), and reticulorumen pH were recorded continuously during the entire experimental period. Total mixed ration samples were collected during the baseline days and analyzed. Samples for nutrient and dry matter (DM) analysis were oven dried at 55°C for 48 h. Dried samples were ground to pass through a 1-mm screen and for analysis of acid detergent fiber (ADF) (AOAC International, 2000: method 973.18), neutral detergent fiber (NDF) with heat-stable α-amylase and sodium sulphite (Van Soest et al., 1991), and crude protein (CP) (N x 6.25; AOAC International 2000: method 990.03; Leco FP-528 Nitrogen Analyzer, Leco, St. Joseph, MI). Nutrient analyses of the offered feed are described in Table 2.1.

2.2.2.1 *Trial 1*

In Trial 1, six primiparous and two multiparous mid-lactation (238.5 ± 54.7 DIM; Mean ± SD) Holstein dairy cows producing 33.4 ± 3.4 kg of milk per day were used. Cows were randomly assigned to either a treatment (PRO_{-4}) or control (CON_{-4}) drench. At 1430 h of d_{-4}, PRO_{-4} cows received an oral 100 mL drench of *Megasphaera elsdenii* NCIMB 41125 containing approximately 2 x 10^8 cfu/mL. At the same time, CON_{-4} cows were orally drenched with 100 mL of distilled water. At the end of the four-week washout period PRO_{-4} and CON_{-4} groups were crossed over and the experimental period started again. Cows that received PRO_{-4} during the first period, received CON_{-4} during the second period and vice versa. During the second period PRO_{-4} and CON_{-4} administration remained on d_{-4}.

2.2.2.2 *Trial 2*

In Trial 2, six primiparous and two multiparous mid-lactation (178.6 ± 35.9 DIM; Mean ± SD) Holstein dairy cows producing 50.9 ± 6.1 kg of milk per day were used. Cows were randomly assigned to either a treatment (PRO_{-1}) or control (CON_{-1}) drench. At 1430 h of d_{-1}, cows in the PRO_{-1} group received 100 mL of an oral drench of *Megasphaera elsdenii* containing approximately 2 x 10^8 cfu/mL. At the same time, CON_{-1} cows were orally drenched with 100 mL of distilled water. At the end of the washout period PRO_{-1} and CON_{-1} groups were crossed over and the experimental period started again. Cows that received PRO_{-1} during the first period, received CON_{-1} during the second period and vice versa. During the second period PRO_{-1} and CON_{-1} administration remained on d_{-1}.

2.2.3 *Statistical analysis*

All statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC). Before analysis, data were checked for normality using the UNIVARIATE procedure in SAS and probability distribution plots. No outliers were detected (data points that were beyond 3 standard deviations from the mean) and no transformations were deemed necessary. Reticulorumen pH, milk yield, milk fat and protein percentages, number of visits to the feeder, DMI, and time spent feeding were summarized by trial day using the MEANS procedure in SAS and expressed as daily means. Milk fat to protein ratio was obtained by dividing milk fat percentage by milk protein percentage and summarized by day. To measure SARA intensity, area under the curve (AUC) analyses were performed using two different reticulorumen pH thresholds, pH < 5.8 and pH < 5.6. Each observed pH value was subtracted from the thresholds and multiplied by the interval between pH

readings. Area under the curve results were then summarized using the MEANS procedure and expressed as mean area under the curve below reticulorumen pH < 5.8 and pH < 5.6. Similarly, time spent below reticulorumen pH 5.6 or 5.8 was summarized by day and expressed as mean time below reticulorumen pH 5.6 or 5.8.

The effect of the *Megasphaera elsdenii* drench was determined by an analysis of variance (ANOVA) using mixed linear models (MIXED procedure) in SAS. The fixed effects in the model included treatment (PRO and CON), crossover sequence, challenge mix intake, milk yield and days in milk at experiment enrollment, lactation, and the interaction between treatment and study day (d_{-4} to d_3). Study day was specified as a repeated measure and cow as subject, using a compound-symmetry structure. Effects with a *p*-value above 0.30 were removed from the model using a stepwise backward elimination process starting with the least contributing effect. The final model for Trial 1 included treatment, crossover sequence, challenge mix intake, milk yield at experiment enrollment, lactation, and the interaction between treatment and study day. The final model for Trial 2 included treatment, challenge mix intake, milk yield at experiment enrollment, and the interaction between treatment and study day. Post-hoc comparison between treatments were carried out to determine differences between the treatments across experimental days utilizing the PDIFF option. P-values were adjusted using the Bonferroni correction. Significance was declared at $P \leq 0.05$, and trends were defined as $0.05 < P \leq 0.10$.

2.3 RESULTS

2.3.1 *Reticulorumen pH*

In Trial 1, PRO_{-4} and CON_{-4} drenches were administered 4 d before the acidosis challenge. Mean reticulorumen pH, area, and length of time below pH 5.8 and pH 5.6 are reported in Table 2.2. Briefly, when looking at the effect of the *Megasphaera elsdenii* drench during the whole experimental period, we found that treatment increased reticulorumen pH ($F_{1,7} = 28.47$; $P < 0.01$; Table 2.2), and reduced AUC ($F_{1,7} = 11.92$; $P = 0.01$; Table 2.2) and time below pH 5.8 ($F_{1,7} = 19.50$; $P < 0.01$; Table 2.2). Also, the treatment affected the AUC ($F_{1,7} = 7.92$; $P = 0.03$; Table 2.2) and time ($F_{1,7} = 10.64$; $P = 0.01$; Table 2.2) below pH 5.6 in the reticulorumen. During Trial 1, *Megasphaera elsdenii* drench positively affected reticulorumen pH dynamics. Treatment cows had greater mean reticulorumen pH on d_{-3}, d_{-1}, d_{+1}, and d_{+2} ($t \geq -2.10$; $P = 0.04$; Figure 2.1 – a). When the pH threshold was set as 5.8, PRO_{-4} cows had lesser area under the curve when compared to control cows on d_0 and d_{+1} ($t \geq 2.12$; $P \leq 0.04$; Figure 2.1 - b). As expected, CON_{-4} cows spent more time below the pH 5.8 threshold on d_{-4}, d_{-3}, d_{-1}, and d_{+1} ($t \geq 2.01$; $P \leq 0.05$; Figure 2.1 - c). When the pH threshold was set as 5.6, CON_{-4} cows had increased AUC d_0 and d_{+1} ($t \geq 2.28$; $P \leq 0.02$; Figure 2.1 - d). In addition, CON_{-4} cows spent more time below the 5.6 pH threshold d_{+1} ($t = 2.53$; $P = 0.01$) and tended to spend more time under the threshold on d_0 ($t = 0.78$; $P = 0.09$; Figure 2.1 - e).

In Trial 2 PRO_{-1} and CON_{-1} drenches were administered on the fourth day of the trial period, the same day the animals had their feed allowance reduced by 50% and one day

before the acidosis challenge day. However, reticulorumen pH dynamics were not affected by treatment ($F_{1,7} = 1.81$; $P \geq 0.22$; Table 2.2).

When analyzing the effect of the treatment by experimental day, we found that PRO_{-1} cows had lesser mean reticulorumen pH on d_{+1} (t = 2.59; $P = 0.01$; Figure 2.2 - a). When the pH threshold was set as 5.8, PRO_{-1} cows tended to have increased AUC on d_{+2}, (t = 1.83; $P = 0.07$; Figure 2.2 - b). No treatment by experimental day differences were found for time below the 5.8 threshold ($t \leq 1.36$; $P \geq 0.18$; Figure 2.2 - c). Likewise, no treatment by day differences in AUC below pH 5.6 ($t \leq 1.63$; $P \geq 0.11$; Figure 2.2 – d). However, PRO_{-1} cows tended to spend less time below pH 5.6 on d_{+1} (t = 1.80 $P = 0.08$; Figure 2.2 – e) when compared to control.

2.3.2 *DMI and feeding behavior*

In Trial 1, treatment affected DMI ($F_{1,7} = 6.12$; $P = 0.04$; Table 2.2), but not feeding behavior ($F_{1,7} = 0.66$; $P = 0.44$; Table 2.2) throughout the experimental period. When analyzing the effect of the treatment by experimental day, there were no significant differences in the number of visits to the feeder ($t \leq 1.26$; $P \geq 0.21$; Figure 2.3 – a) or time spent feeding ($t \leq 0.79$; $P \geq 0.43$; Figure 2.3 – b) between PRO_{-4} and CON_{-4}. However, PRO_{-4} cows had significantly greater DMI on d_{+1} (t = 2.61; $P = 0.01$; Figure 2.4).

In Trial 2, DMI and number of visits to the feeder were not affected by treatment ($F_{1,7} \leq 0.19$; $P \geq 0.67$; Table 2.2). However, PRO_{-1} cows spent more minutes per day feeding compared to control cows ($F_{1,7} = 13.25$; $P < 0.01$; Table 2.2). When looking at the effects of treatment by experimental day, PRO_{-1} cows tended to visit the feeder less often on d_{-3}, and more often on d_0 ($t \geq 1.69$; $P \leq 0.09$; Figure 2.5 – a). Also, CON_{-1} cows spent

less time feeding on d_{-4} and d_{-3} ($t \geq 2.51$; $P \leq 0.01$; Figure 2.5 – b), and tended to spend less time feeding on d_{-2} ($t = 1.91$; $P = 0.06$; Figure 2.5 – b). Additionally, PRO_{-1} cows had greater DMI d_0 ($t = 2.86$; $P < 0.01$; Figure 2.6) and tended to have decreased intake on d_{+3} ($t = 1.89$; $P = 0.06$; Figure 2.6)

2.3.3 *Milk yield and components*

In Trial 1, during the whole experimental period milk production was affected by treatment with PRO_{-4} cows having greater daily milk yield compared to CON_{-4} cows ($F_{1,7} = 10.80$; $P = 0.01$; Table 2.2). The differences in reticulorumen pH dynamics and feed intake might have also influenced the milk components, where milk protein percentage was greater for PRO_{-4} cows ($F_{1,7} = 8.08$; $P = 0.03$; Table 2.2) and PRO_{-4} cows tended to have lesser milk fat percentage in comparison with CON_{-4} cows ($F_{1,7} = 4.86$; $P = 0.06$; Table 2.2). Additionally, treatment influenced milk fat to protein ratio with PRO_{-4} cows having a lesser milk fat to protein ratio compared to CON_{-4} ($F_{1,7} = 11.37$; $P = 0.01$; Table 2.2).

When looking at the effects of treatment by experimental day, PRO_{-4} cows tended to produce more milk on d_{-3} and d_{+2} ($t \leq 1.88$; $P = 0.06$; Figure 2.7 – a). Additionally, CON_{-4} tended to have greater milk fat percentage compared to PRO_{-4} cows on d_{-4} ($t = 1.76$; $P = 0.08$; Figure 2.7 – b), and had greater milk fat on d_{-1}, ($t = 1.95$; $P = 0.05$; Figure 2.7 – b). Milk protein percentage tended to be greater for PRO_{-4} cows on d_{-3}, d_{-1}, and d_{+2} ($t \leq 1.72$; $P \leq 0.09$; Figure 2.7 – c). Consequently, CON_{-4} cows had greater fat to protein ratio on d_{-1} ($t = 2.40$; $P = 0.02$; Figure 2.7 – d) and tended to have greater ratio on d_{+2} ($t = 1.85$; $P = 0.07$; Figure 2.7 – d).

In Trial 2, when looking at the whole experimental period, milk production and components were not affected by treatment ($F_{1,7} \leq 2.02$; $P \geq 0.20$; Table 2.2). When looking at the effect of the treatment by experimental day, PRO_{-1} cows produced more milk on d_0 ($t = 2.31$; $P = 0.02$; Figure 2.8 – a) and tended to produce more milk on d_{-3}, ($t = 1.66$; $P = 0.10$ Figure 2.8 – a). Milk fat percentage was greater in CON_{-1} cows on d_0 ($t = 2.01$; $P = 0.05$; Figure 2.8 – b). On the other hand, PRO_{-1} cows had greater milk protein percentage on d_0 ($t = 2.54$; $P = 0.01$; Figure 2.8 – c). Consequently, CON_{-1} cows had greater fat to protein ratio on d_0 ($t = 3.05$; $P < 0.01$; Figure 2.8 – d).

2.4 DISCUSSION

In this study, we tested the efficacy of an oral drench containing *Megasphaera elsdenii* in lactating cows under SARA risk. This study builds upon previous works showing benefits of utilizing a *Megasphaera elsdenii* oral drench on ruminal environment (Aikman et al., 2011), feed intake (Drouillard, 2004), and milk production (Aikman et al., 2011). However, the effects of drenching cows with *Megasphaera elsdenii* on feeding behavior of lactating dairy cows have not been previously investigated.

We found that drenching cows with *Megasphaera elsdenii* 4 d before an acidosis challenge improved reticulorumen pH throughout the experimental period. However, no improvement was seen when cows were drenched 1 d before an acidosis challenge. In Trial 1, cows that were drenched with *Megasphaera elsdenii* had greater mean daily reticulorumen pH following the acidosis challenge. In addition, CON_{-4} cows spent almost 3 h/d with reticulorumen pH below 5.6 while PRO_{-4} cows spent close to 1 h below the same threshold. These results indicate that CON_{-4} cows were close to having SARA throughout

the study period, given that Plaizier et al. (2008) defined SARA as when rumen pH stays below 5.6 for more than 3 h/d. A reduced AUC means that the pH drop experienced by treatment cows were less severe compared to control cows. Similarly, Henning et al. (2010b) found that lambs drenched with *Megasphaera elsdenii* suffered fewer pH drops compared to control lambs. Aikman et al. (2011) reported that AUC for pH 5.6 or 6.0 was lesser in cows drenched with *Megasphaera elsdenii*, although treatment was not found to be statistically significant.

The lack of long-lasting effects of *Megasphaera elsdenii* drenching in PRO_{-1} cows may be due to the short time period between drenching and the acidosis challenge. Because the drench was administered one day before the acidosis challenge and the same day that cows had their feed allowance reduced in half, we hypothesize that there was not enough time and substrate to allow the introduced *Megasphaera elsdenii* to become established in the rumen. In one of the experiments reported by Weimer et al. (2015), the author hypothesizes that ruminal conditions might be suboptimal for development of the bacteria when dosing cows before feeding. In that same study, *Megasphaera elsdenii* populations returned to very low baseline levels within 24 h of dosing when the drench was administered before feeding. Henning et al. (2010b) reported that viable *Megasphaera elsdenii* populations can be established in 4 to 5 d, and that drenched cows tended to have greater *Megasphaera elsdenii* populations on the first 2 d after dosing. However, it appears that the time of drench administration might influence *Megasphaera elsdenii* establishment and drench efficacy, which was not tested in this study and should be further investigated.

As rumen pH increased, treatment influenced feed intake as PRO_{-1} cows had a higher DMI over the experimental period. To our knowledge, the present study is the first to look

at feeding behavior in lactating cows drenched with *Megasphaera elsdenii.* Our results agree with previous studies in lambs and beef cattle; for example Henning et al. (2010b) reported that lambs and steers drenched with *Megasphaera elsdenii* showed increased feed intake compared to non-drenched individuals. Also, Drouillard (2004) reported that feedlot cattle tended to maintain greater and more consistent feed intake after being drenched with *Megasphaera elsdenii*. However, we cannot conclude that feeding behavior (number of visits to the feeder and feeding time), was affected by drenching the cows with a live culture of *Megasphaera elsdenii*, as the only significant differences between treatments were found in Trial 2 and occurred before the day treatment was administered to the animals. Therefore, future research should investigate other behavioral impacts of *Megasphaera elsdenii* drench in dairy cattle.

Our results agree with previous studies; for example daily milk yield increase was observed in high yielding lactating dairy cows fed a high-starch diet drenched with *Megasphaera elsdenii* (Aikman et al., 2009). Additionally, Aikman et al. (2011) observed a 2.4 kg/d numerical, but not statistically significant, increase in milk yield in cows drenched with *Megasphaera elsdenii.* Moreover*,* Henning et al. (2010b) reported that lambs and steers drenched with *Megasphaera elsdenii* had increased feed intake and average daily gains compared to control animals. With the increased milk protein and decreased milk fat, it was expected that treatment cows would have decreased fat to protein ratio. This is in contrast to Aikman et al. (2008) that cows receiving *Megasphaera elsdenii* had decreased milk protein percentages. On the other hand, the same authors reported a tendency for cows drenched with *Megasphaera elsdenii* to have reduced milk fat content. Some authors have associated the increase of ruminal *Megasphaera elsdenii* to decrease in

milk fat concentration (Palmonari et al., 2010, Weimer et al., 2015) because some strains of *Megasphaera elsdenii* produce *trans*-10,*cis*-12, a conjugated linoleic acid capable of inhibiting milk fat synthesis in the mammary gland (Kim et al., 2002, Bauman and Griinari, 2003).

The *Megasphaera elsdenii* drench has the potential to be used as a strategic management tool to minimize ruminal acidosis and improve ruminal resiliency during the transition period or other nutritional challenging events in the dairy cow life. Drastic dietary changes between the dry period and parturition can increase the risk of ruminal acidosis (Nocek, 1997). Additionally, with the recent availability and adoption of in-line milk analyzers, farmers can utilize milk yield and components data to identify cows at risk of acidosis and possibly make management decisions (Rotz et al., 2003), such as utilizing a *Megasphaera elsdenii* drench. Future research should focus on the strategic uses of the drench and investigate how the establishment of the *Megasphaera elsdenii* population relates to rumen pH and health.

2.5 CONCLUSIONS

The results from this experiment indicate that there might be benefits of utilizing a *Megasphaera elsdenii* drench in lactating dairy cows. We found that the drench positively influenced reticulorumen pH dynamics, and increased feed intake and acidosis resilience, which consequently could have affected milk production and components. There was not sufficient evidence to indicate that drenching cows with *Megasphaera elsdenii* affects feeding behavior. Importantly, we found that the timing of drench administration may be an important factor for drench effectiveness and future research should investigate the

dynamics of *Megasphaera elsdenii* population establishment relationship with rumen pH and health.

Table 2.1 Ingredients and chemical composition of the diets fed during Trial 1, Trial 2, and subacute acidosis challenge for cows (n = 8) drenched with distilled water (control) or *Megasphaera elsdenii* before an acidosis challenge.

Item	Trial 1	Trial 2	SARA challenge mix[1]
Ingredient (g/kg of DM)			
Grain mix[2]	463.0	471.0	
Corn silage	252.0	289.0	
Alfalfa silage	175.0	0.0	
Cottonseed	64.0	65.0	
Mineral mix[3]	24.0	17.0	
Alfalfa hay	22.0	158.0	
Chemical Composition (g/kg of DM)			
DM	569.4 ± 34.2	499.4 ± 35.3	699.4 ± 7.1
CP	147.0 ± 6.4	152.9 ± 4.9	131.4 ± 5.4
aNDF	314.1 ± 39.5	268.9 ± 17.9	184.1 ± 8.6
ADF	204.6 ± 34.4	171.1 ± 10.2	102.2 ± 7.5
Starch	270.2 ± 34.5	286.4 ± 22.0	418.8 ± 11.8
Ether Extract	36.4 ± 4.0	34.5 ± 5.3	34.2 ± 7.2
NFC	448.6 ± 32.4	475.7 ± 16.6	606.5 ± 6.3
Ash	74.5 ± 8.8	84.9 ± 3.5	60.0 ± 3.5
Ca	11.1 ± 2.2	11.0 ± 0.6	5.7 ± 0.4
P	6.2 ± 0.2	6.2 ± 0.4	5.0 ± 0.6

[1] The SARA challenge mix consisted of 2 kg of rolled barley, 2 kg of ground wheat and 0.9 kg, and 4.3 kg of TMR.

[2] The grain mix contained (%) ground corn (48.7), soft wheat middlings (14.5), dry corn gluten feed (11.7), SoyPlus (7.4), ground soybean hulls (5.5), soybean meal (4.9), calcium carbonate (2.3), molasses (1.7), sodium bicarbonate (1.6), white salt (0.8), magnesium oxide (0.7), urea (0.1), mineral mix[2] (0.1).

[3] The mineral supplement had the following composition: vitamin A (2,141,457 IU/kg), vitamin D3 (535,392 IU/kg), vitamin E (8,103 IU/kg), Zn (16,506 mg/kg), Mn (15,020 mg/kg), Cu (2,720 mg/kg), I (351 mg/kg), Co (239 mg/kg), Se (106 mg/kg), Ca (109 g/kg), P (2 g/kg), Mg (1.8 g/kg), and Na (0.5 g/kg).

Table 2.2 Least square means (± SEM) of reticulorumen pH dynamics, DMI, feeding behavior, and milk yield and composition for dairy cows (n=8) drenched with distilled water (control) or *Megasphaera elsdenii* 4 days (Trial 1) and 1 day (Trial 2) before an acidosis challenge.

Item	Treatment		SEM	*P-value*
	Control	*Megasphaera elsdenii*		
Megasphaera elsdenii 4 days before an acidosis challenge				
Reticulorumen pH Dynamics				
Mean reticulorumen pH average	6.11	6.23	0.05	< 0.01
Area below pH 5.8 (min x pH/d)	81.38	20.85	27.35	0.01
Area below pH 5.6 (min x pH/d)	37.31	6.77	13.89	0.03
Time below pH 5.8 (h/d)	4.68	1.82	1.42	< 0.01
Time below pH 5.6 (h/d)	2.65	0.64	0.89	0.01
DMI and Feeding Behavior				
DMI (kg/d)	21.43	23.50	1.44	0.04
Visits to the feeder (visits/d)	27.99	29.34	1.65	0.44
Time spent feeding (min/d)	191.65	200.62	27.72	0.58
Milk yield and Components				
Milk yield (kg/d)	26.07	28.75	1.62	0.01
Milk fat (%)	4.09	3.97	0.08	0.06
Milk protein (%)	3.19	3.28	0.15	0.02
Fat to protein ratio	1.30	1.22	0.06	0.01
Megasphaera elsdenii 1 day before an acidosis challenge				
Reticulorumen pH Dynamics				
Mean reticulorumen pH average	6.24	6.21	0.07	0.22
Area below pH 5.8 (min x pH/d)	21.54	36.26	11.60	0.22
Area below pH 5.6 (min x pH/d)	19.12	17.01	11.17	0.75

Table 2.2 Continued

Item	Treatment		SEM	*P-value*
	Control	*Megasphaera elsdenii*		
Time below pH 5.8 (h/d)	2.61	3.20	0.79	0.35
Time below pH 5.6 (h/d)	1.66	1.31	0.63	0.32
DMI and Feeding Behavior				
DMI (kg/d)	22.80	22.88	0.83	0.86
Visits to the feeder (visits/d)	37.42	35.98	4.86	0.67
Time spent feeding (min/d)	282.66	378.19	53.24	< 0.01
Milk yield and Components				
Milk yield (kg/d)	34.26	34.97	0.95	0.20
Milk fat (%)	3.78	3.82	0.04	0.37
Milk protein (%)	3.21	3.22	0.08	0.58
Fat to protein ratio	1.18	1.19	0.02	0.50

Figure 2.1 Reticulorumen pH dynamics differences expressed as least square means ± SEM by experimental day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 4 days before the acidosis challenge for: a) reticulorumen pH average, b) area below pH 5.8, c) time below pH 5.8, d) area below pH 5.6, e) time below pH 5.6.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.1 A)

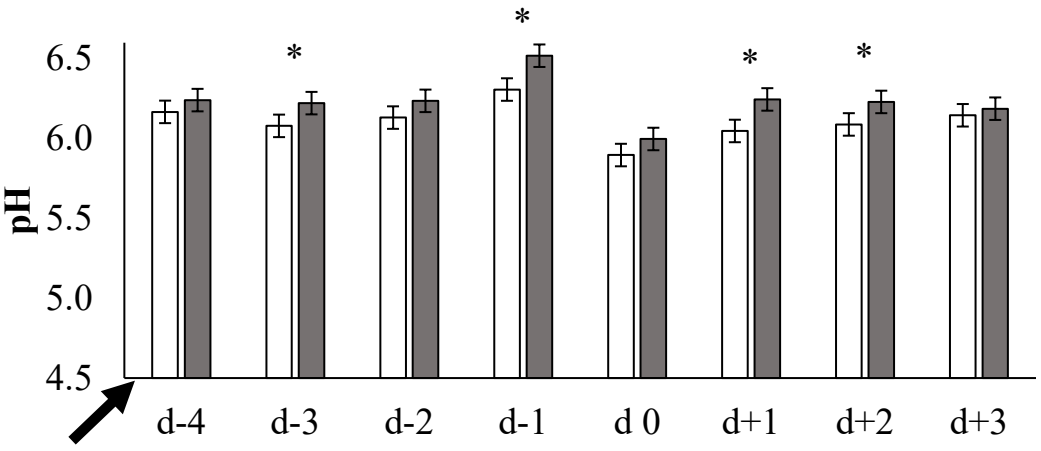


Figure 2.1 B)

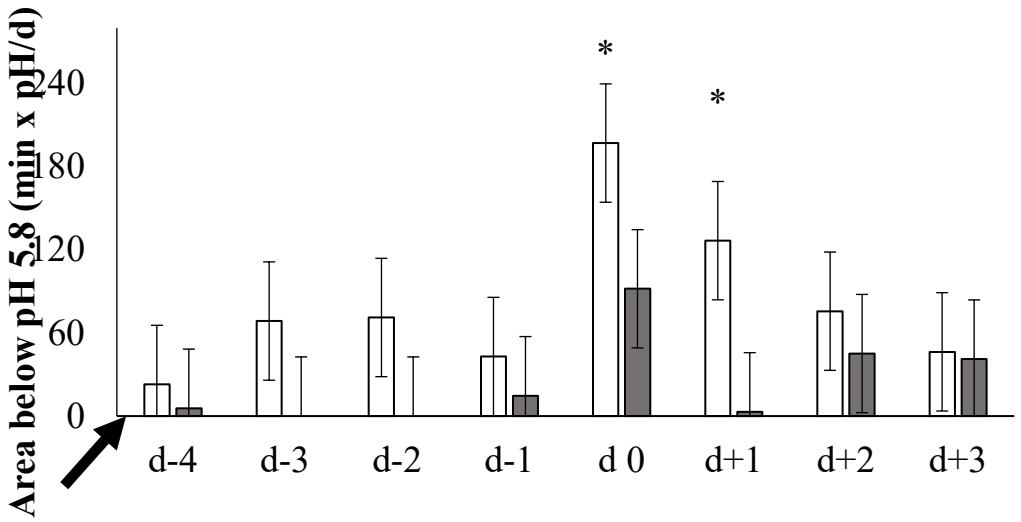


Figure 2.1 C)

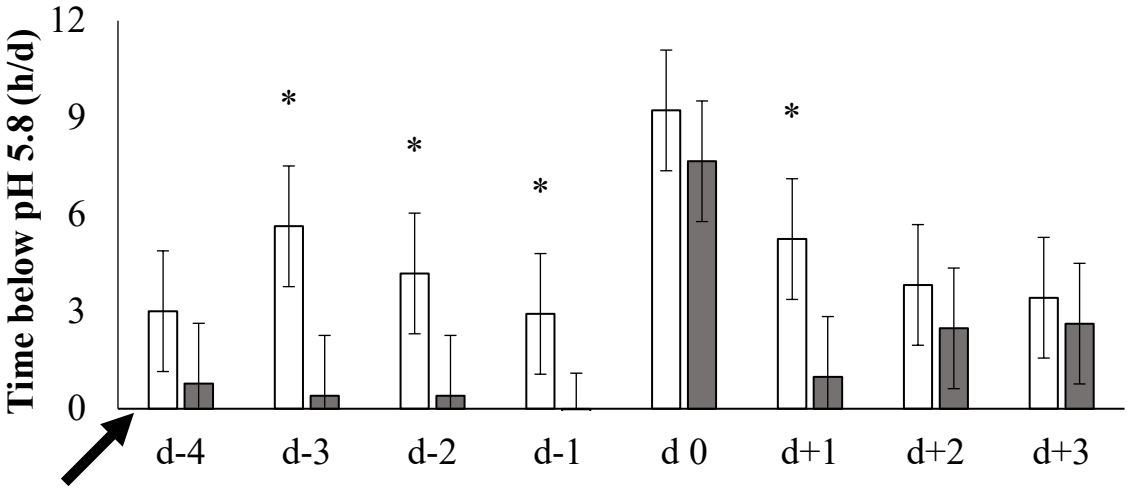


Figure 2.1 D)

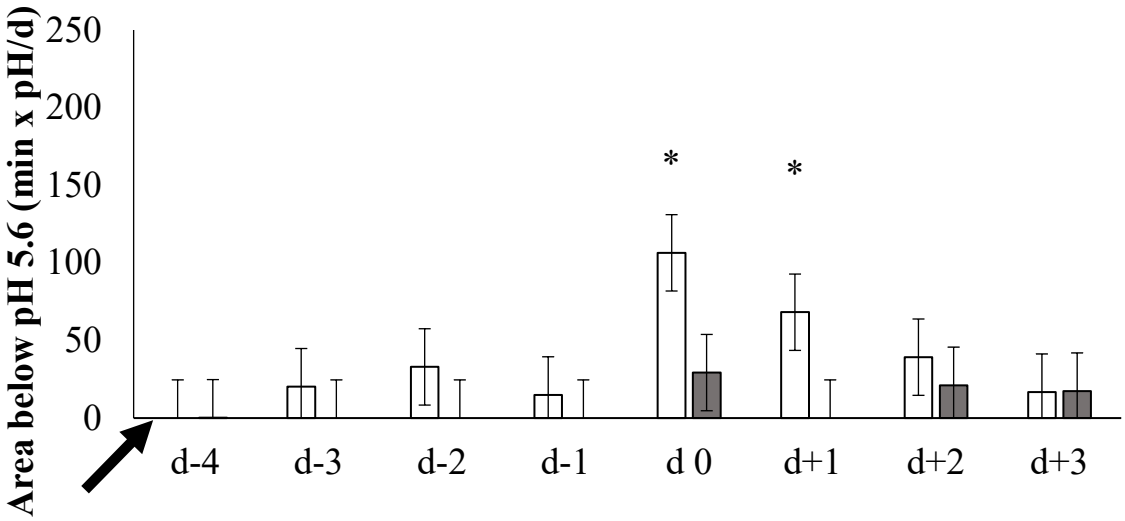

Area below pH 5.6 (min x pH/d)
250
200
150
100
50
0
*
*
d-4
d-3
d-2
d-1
d 0
d+1
d+2
d+3

Figure 2.1 E)

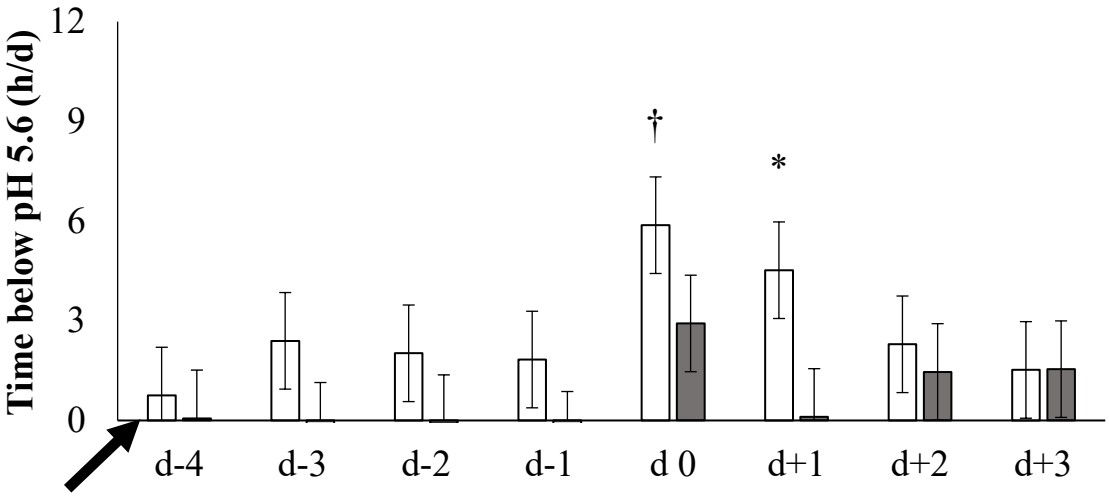


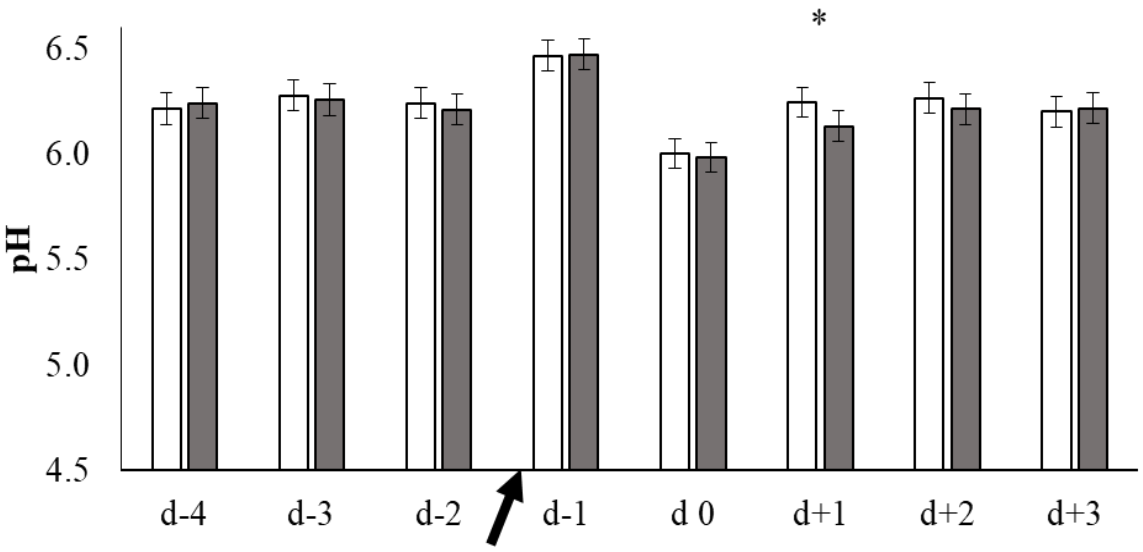
Figure 2.2 Reticulorumen pH dynamics differences expressed as least square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 1 day before the acidosis challenge for: a) reticulorumen pH average, b) area below pH 5.8, c) time below pH 5.8, d) area below pH 5.6, **e)** time below pH 5.6.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.2 A)



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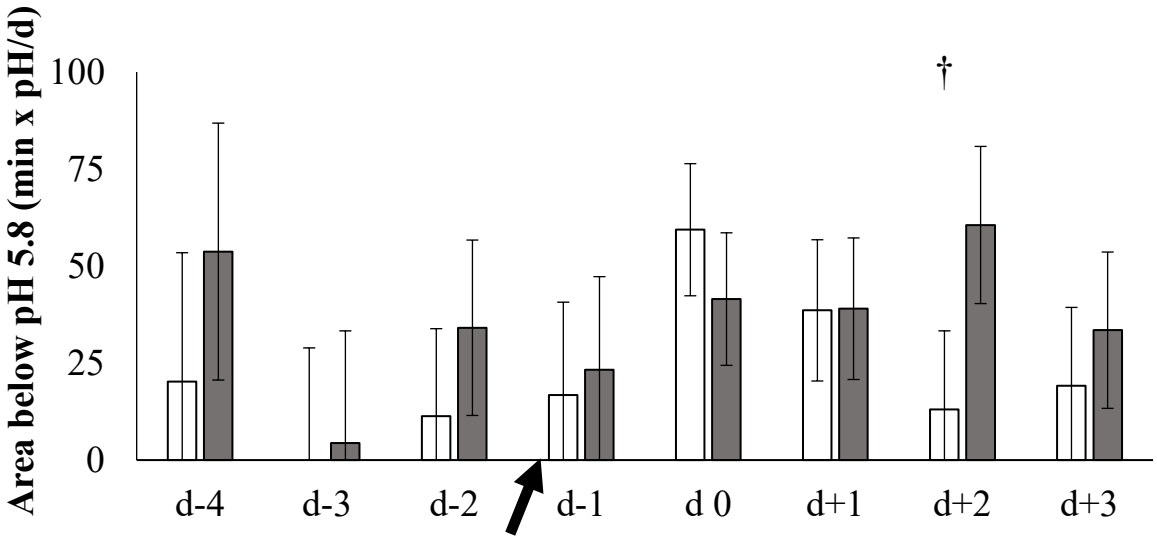


Figure 2.2 C)

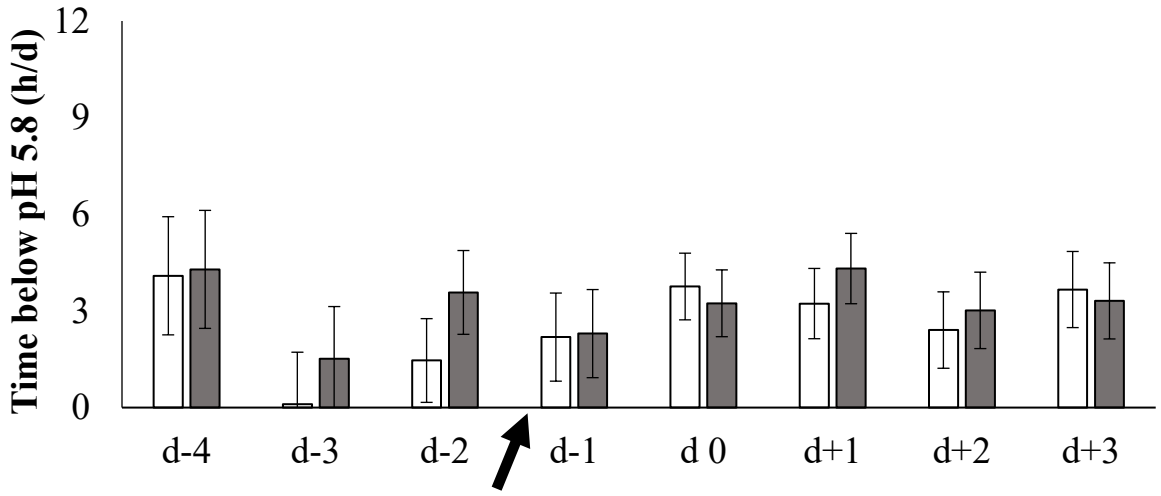


Figure 2.2 D)

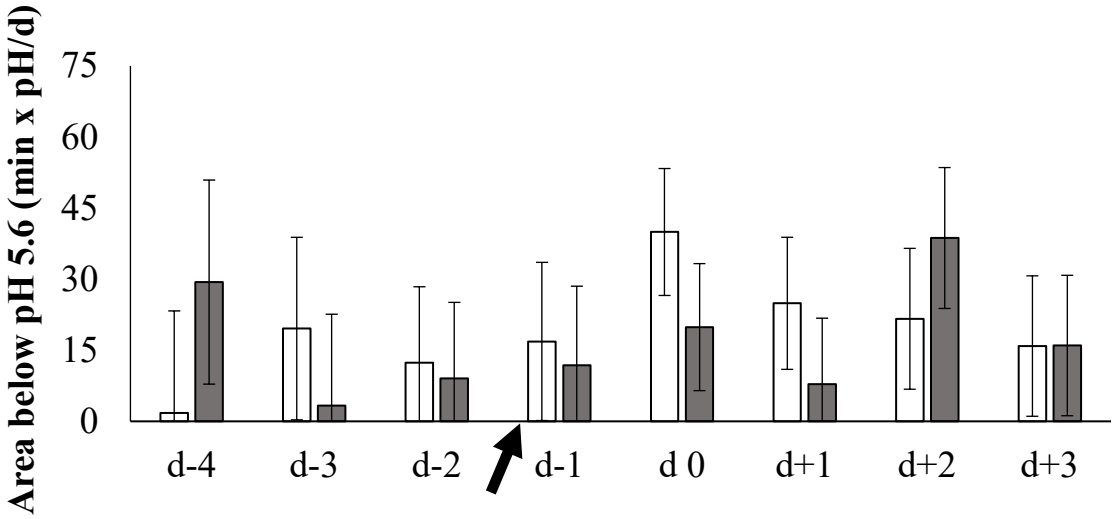


Figure 2.2 E)

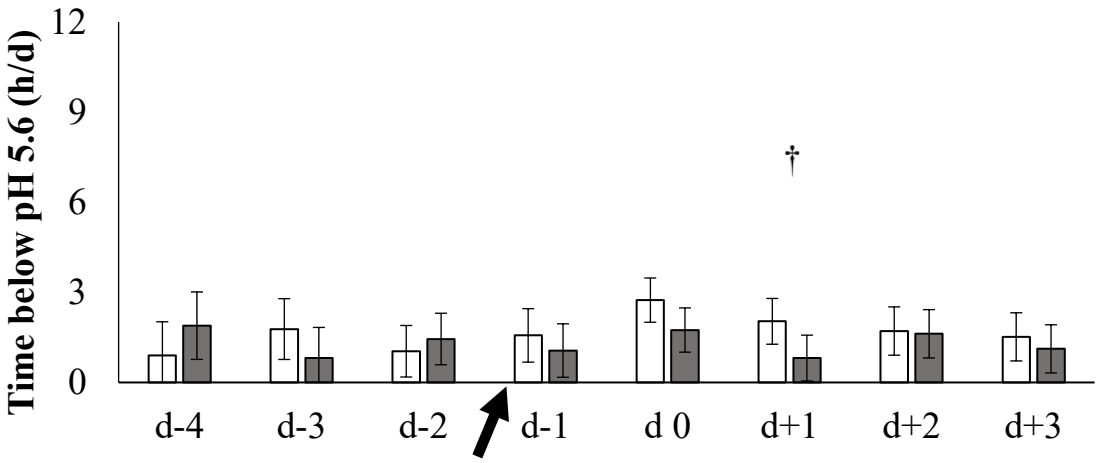


Figure 2.3 Feeding behavior differences expressed as least Square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 4 days before the acidosis challenge for: a) number of visits to the feeder, b) feeding time.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.3 A)

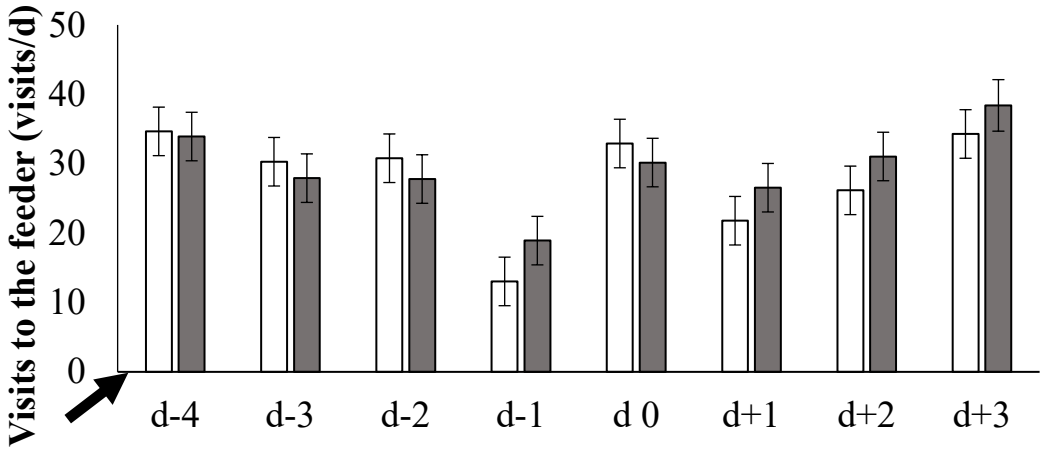


Figure 2.3 B)

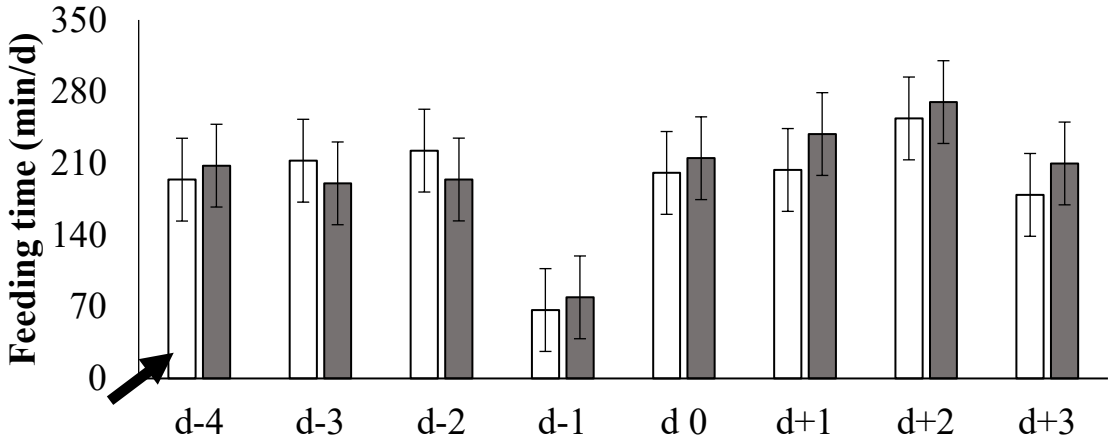


Figure 2.4 Dry matter intake differences expressed as least Square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 4 days before the acidosis challenge.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

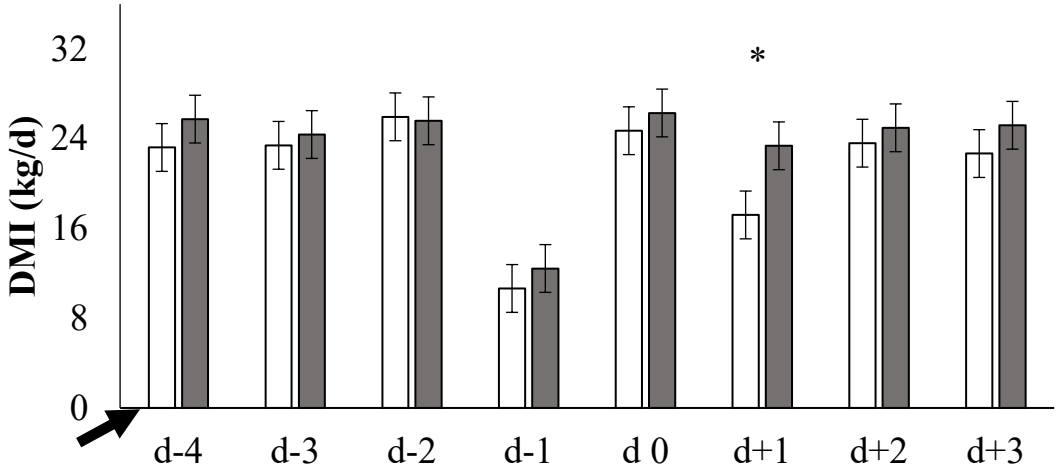


Figure 2.5 Feeding behavior differences expressed as least square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 1 day before the acidosis challenge for: a) number of visits to the feeder, b) feeding time.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.5 A)

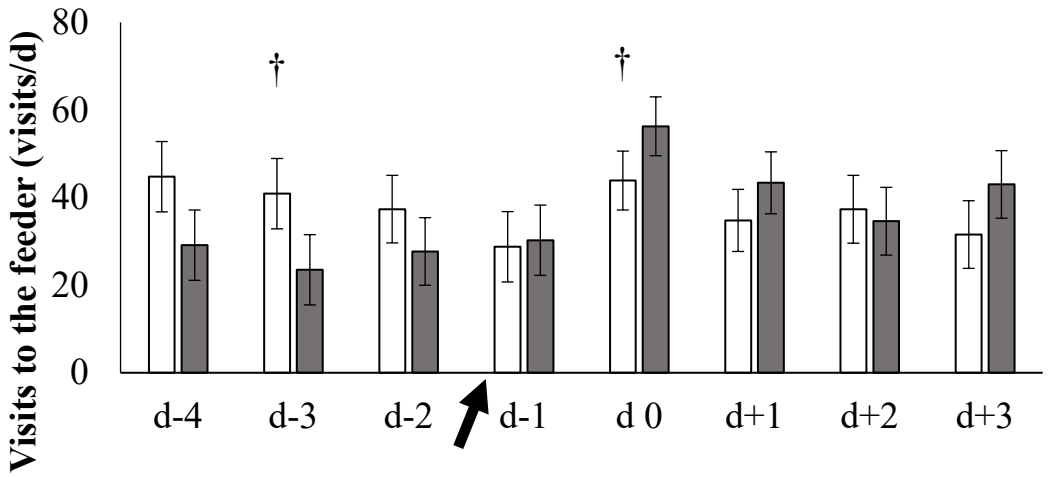


Figure 2.5 B)

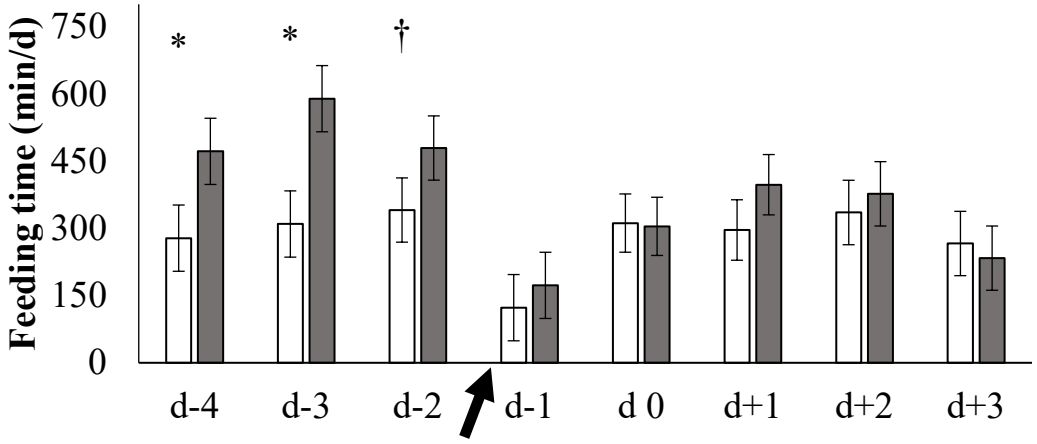


Figure 2.6 Dry matter intake differences expressed as least Square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 1 day before the acidosis challenge.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

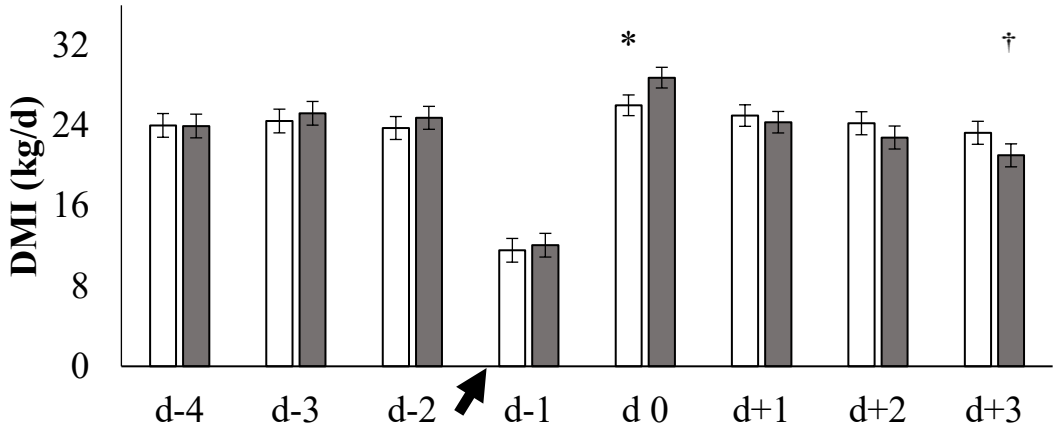


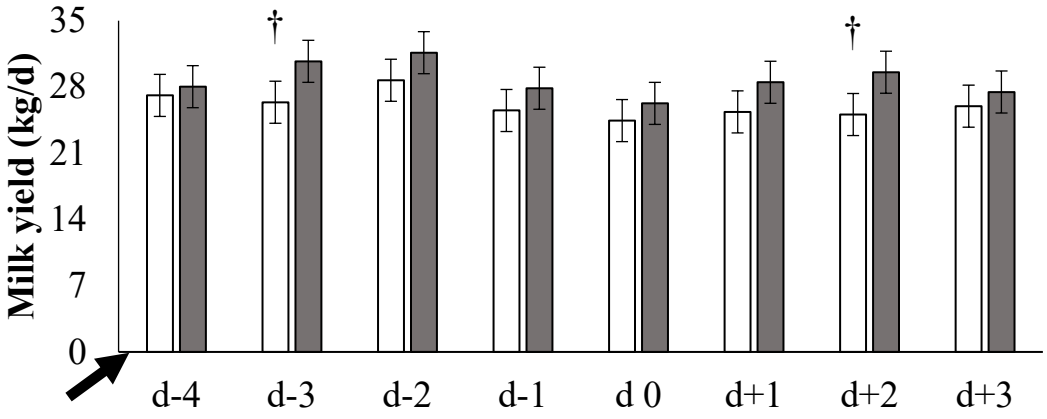
Figure 2.7 Milk yield and components differences expressed as least Square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 4 days before the acidosis challenge for: a) milk yield, b) milk fat, c) milk protein, d) milk fat to protein ratio.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.7 A)



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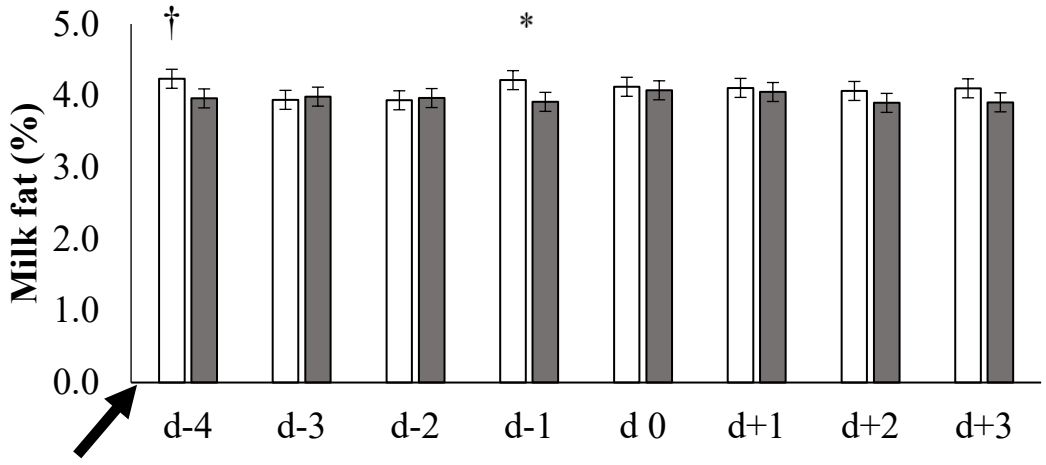

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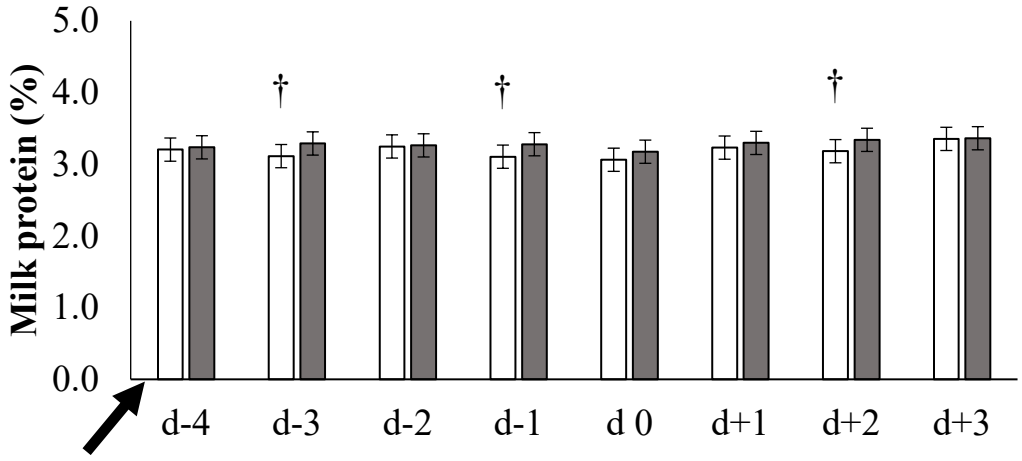

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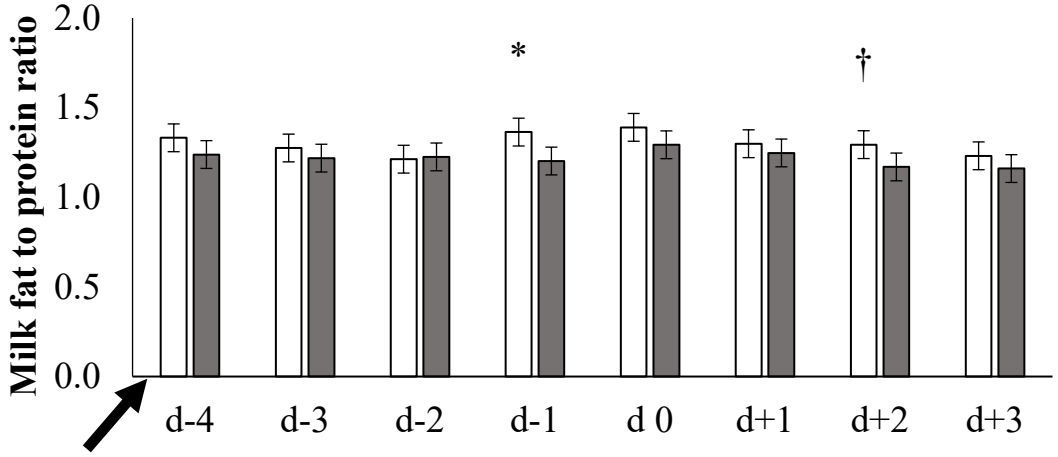


Figure 2.8 Milk yield and components differences expressed as least Square means ± SEM by day relative to an acidosis challenge (d0) in cows drenched with distilled water (white) or *Megasphaera elsdenii* (gray) 1 day before the acidosis challenge for: a) milk yield, b) milk fat, c) milk protein, d) milk fat to protein ratio.

→ Indicates time of drenching.

* Indicates that treatments differed on that day ($P < 0.05$).

† Indicates that treatments tended to differ between treatments on that day ($P < 0.10$).

Figure 2.8 A)

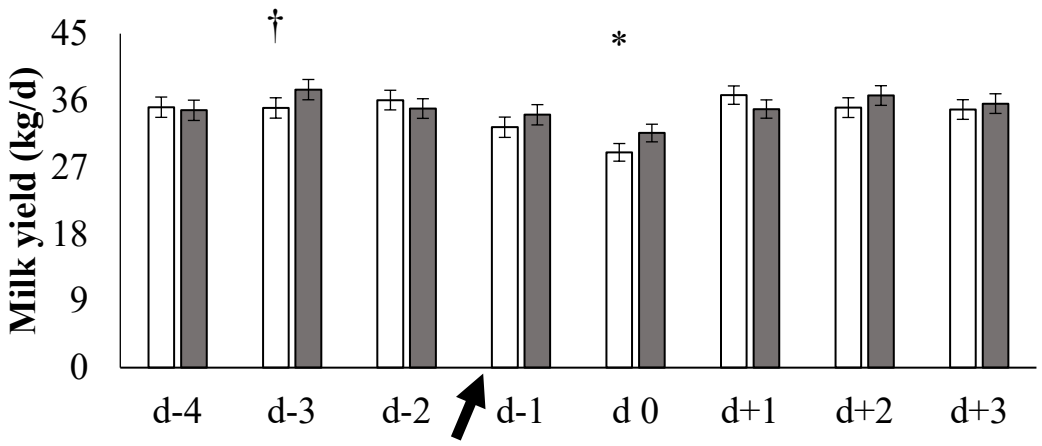


Figure 2.8 B)

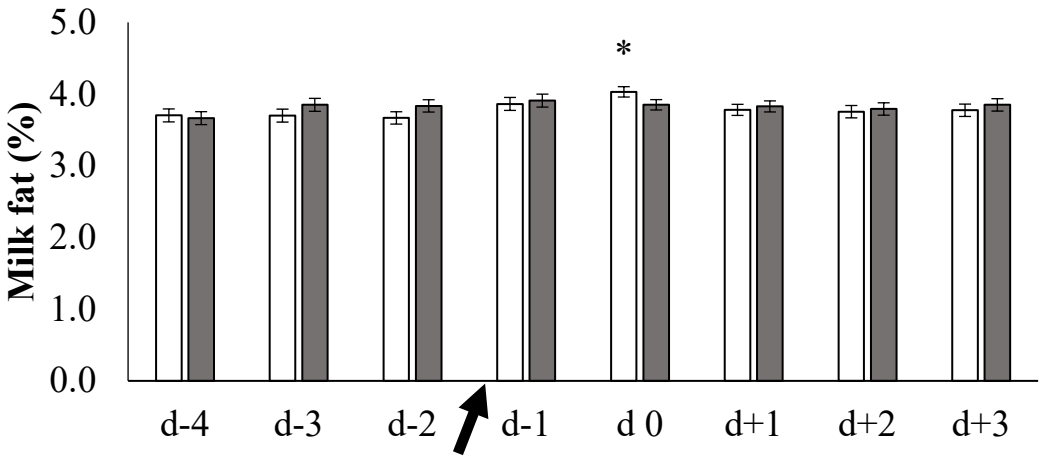


Figure 2.8 C)

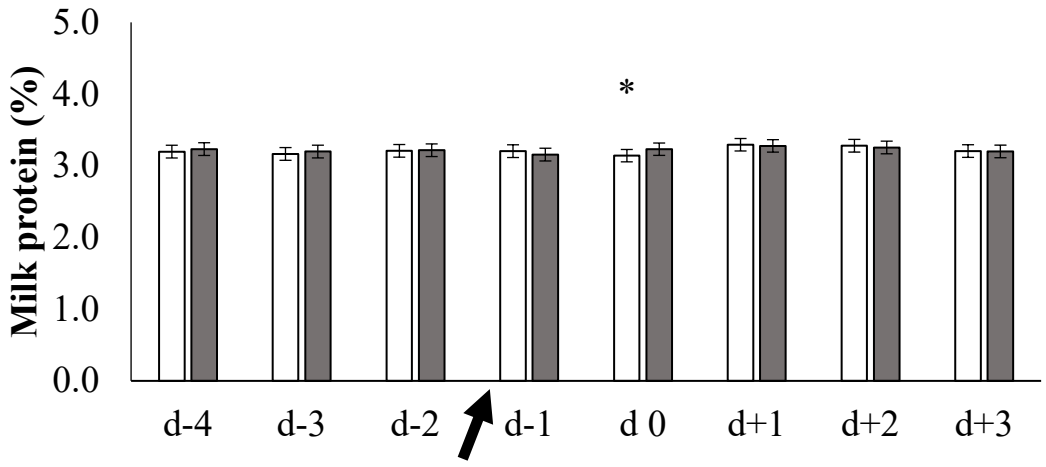
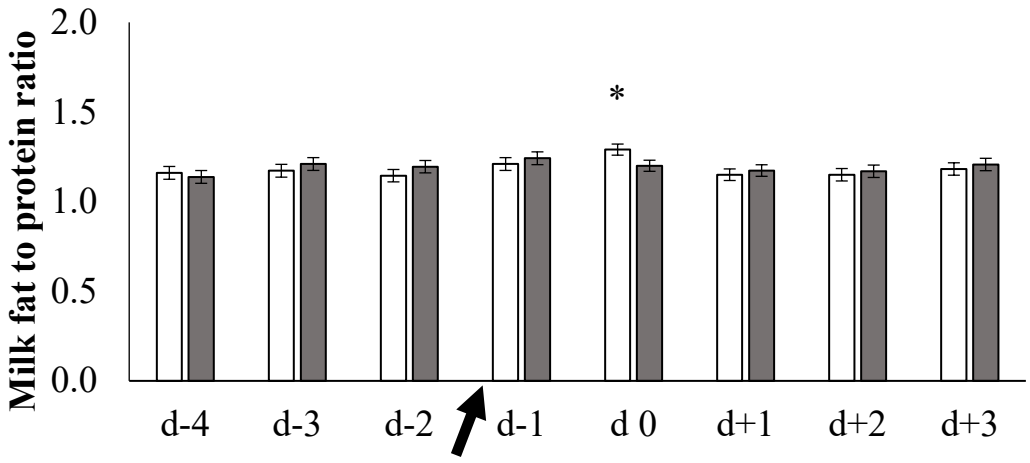


Figure 2.8 D)



CHAPTER 3. EFFECTS OF A *MEGASPHAERA ELSDENII* ORAL CAPSULE ON THE DEVELOPMENT OF DAIRY-BEEF CROSSBRED CALVES. PART I: PERFORMANCE AND FEEDING BEHAVIOR PATTERNS

3.1 INTRODUCTION

Ruminal health is fundamental to calf growth and development, and one of the many maladies affecting calves ruminal health is subacute ruminal acidosis (SARA). Subacute ruminal acidosis is a metabolic disorder characterized by low reticulorumen pH (i.e. pH < 5.6 or pH < 5.8 for more than 180 min) that can affect the animal's physiology, intake, feeding and resting behavior, and performance (see review by Plaizier et al., 2008). Although vastly studied in adult cattle, there is limited research on the effects and prevention of SARA in calves. Previous research has shown that reticulorumen pH in dairy calves is lower than in adult cattle, ranging from 5.0 to 6.0 (Gentile et al., 2004, Suarez-Mena et al., 2016, Yohe et al., 2018). In addition, dairy calves are also prone to decreases in reticulorumen pH, especially during weaning (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020).

A recent study by Gelsinger et al. (2020) reported that calves with induced SARA had decreased solid feed dry matter intake (DMI) and body weights. Likewise, Li et al. (2019) reported a significant impact of SARA on solid feed DMI and body weight starting at four and five weeks of age, respectively. Gut dysfunctions such as SARA have been associated with the display of non-nutritive oral behaviors in ungulates (Bergeron et al., 2006). Non-nutritive behaviors such as tongue rolling, licking, and oral manipulation are normally displayed by calves (Horvath and Miller-Cushon, 2017). However, the excessive display of these non-nutritive oral behaviors has been associated with poor animal welfare

(Mason and Latham, 2004). In addition to the negative effects on solid feed DMI, performance, and behavior, SARA might affect reticulorumen volatile fatty acid (VFA) dynamics (Gelsinger et al., 2020) and development (Li et al., 2019, Gelsinger et al., 2020) in calves. Persistence of SARA can also lead to metabolic acidosis and death in calves (Gentile et al., 2004).

In dairy cows, one of the many prevention methods for acidosis is the use of probiotics containing lactate-utilizing bacteria (Nocek et al., 2002). For example, *Megasphaera elsdenii* is one of the lactate-utilizing bacteria that can be utilized as a probiotic for SARA prevention. *Megasphaera elsdenii* is a naturally occurring rumen microorganism known for utilizing lactate and stabilizing ruminal pH (Counotte et al., 1981b, Nagaraja and Titgemeyer, 2007c). In fact, strains of *Megasphaera elsdenii* have been patented and can now be used as SARA control tools (Leedle et al., 1990; Horn et al., 2009). In a recent study with mid-lactation cows, Mazon et al. (2020) reported that challenged cows receiving a probiotic containing *Megasphaera elsdenii* experienced shorter and less intense acidosis periods compared to control cows. In recent reviews, Alawneh et al. (2020) and Cangiano et al. (2020) reported that probiotic supplementation in calves generally yields positive effects, especially when animals are subject to stress. Furthermore, providing calves with probiotics around weaning is considered a strategic intervention to mitigate the reticulorumen exposure to events that can negatively impact calf health and performance (Cangiano et al., 2020). Besides weaning, calves are also likely to benefit from early-life nutritional interventions with probiotics. The rationale behind early-life administration of probiotics to calves consists in the introduction of beneficial microorganisms into the reticulorumen that will pre-empt the reticulorumen environment,

proliferate, and bring long-term benefits for the calf (Arshad et al., 2021, Du et al., 2023). Therefore, there is an opportunity to supplement probiotics, such as *Megasphaera elsdenii* both in early-life as well as around weaning as a SARA prevention tool for calves.

The current literature on the supplementation of probiotics containing *Megasphaera elsdenii* to calves is still limited, focuses on early-life supplementation, and shows contrasting results. Muya et al. (2015) reported that calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age had greater DMI, greater weaning weight, and tended to have greater average daily gain compared to control calves. However, Yohe et al. (2018) did not observe any effects of *Megasphaera elsdenii* oral supplementation on body weight or DMI in dairy calves. On the other hand, supplementation of *Megasphaera elsdenii* in beef cattle has yielded positive results, increasing meat quality in early weaned steers (DeClerck et al., 2020b) and ADG in beef cows (DeClerck et al., 2020a).

Thus, the objective of this study was to evaluate the effects of an oral probiotic capsule containing a live culture of *Megasphaera elsdenii* NCIMB 41125 (Lactipro FLX Calf, MS Biotec) on the solid feed DMI, performance, and behavioral patterns (eating, drinking, and non-nutritive oral behaviors) of dairy-beef crossbred calves under SARA risk throughout the rearing period. We hypothesized that calves receiving the early-life intervention with *Megasphaera elsdenii* would have greater solid feed DMI, which could possibly increase their ADG, and the time they spent eating and drinking water. Furthermore, we hypothesized that calves receiving the early-life intervention with *Megasphaera elsdenii* spent less time performing non-nutritive oral behaviors compared to control.

3.2 MATERIALS AND METHODS

3.2.1 *Animal Housing and Diet*

This study was conducted at the University of Kentucky Large Animal Unit between August 2020 and April 2021, under Institutional Animal Care and Use Committee Protocol number 2019-3156. The sample size was calculated based on previous work (Muya et al., 2015, Muya et al., 2017) and a review of available calf performance records from the University of Kentucky Large Animal Unit. A power analysis was calculated to detect a difference of 0.20 kg/d of ADG with a standard deviation of 0.15 kg and an estimated population mean ADG of 0.8 kg/d. Using these values, nine calves were needed per treatment group to achieve a power of 80% with an α of 0.05. To account for potential animal or data loss, at least 10 animals were enrolled in each treatment group.

Thirty-one dairy-beef crossbred calves (Holstein x Angus; 45.3 ± 7.1 kg; 8.2 ± 2.0 d old) were enrolled in a blinded 76-day randomized trial. Calves were acquired from a commercial dairy farm and transported to the University of Kentucky Large Animal Unit in Lexington Kentucky. Upon birth at the commercial farm, calves were fed pasteurized maternal colostrum within six hours of birth. Calves were individually housed outdoors in hutches and fed 6 L of pasteurized milk divided into two meals at approximately 0700 and 1500 h.

At the University of Kentucky Large Animal Unit, calves were individually housed indoors in an environmentally controlled room (20.9 ± 0.5^{o} C and 79.4 ± 5.0% relative humidity). Calf pens were 6.5 ± 0.3 m^2 with solid side walls and lined with rubber mattress flooring. Calves had *ad libitum* access to water and a pelletized calf starter (Table 3.1; Special Calf Starter and Grower, Bagdad Roller Mills Inc, Bagdad, KY) provided via

buckets. Calf starter buckets were replenished daily at 0830 h and once weekly each bucket was emptied, thoroughly washed, and the calf starter was completely replaced. The calves were bottle fed, receiving 7 L/d of milk replacer (Cow's Match Warm Front, Land O Lakes, MN; 150 g of milk replacer for 1L of water heated to 40 ^{o}C; 13.04 % DM; 27 % CP; 10 % crude fat, and 40 % lactose) divided into two equal meals at approximately 0800 and 1730h until day 41 of the study. On day 42, calves had their milk allowance reduced to 3.5 L/d of the same solution divided into two equal meals. If a calf was unable to drink the whole contents of the bottle, researchers would manually assist the calf to access the bottle. If the calf refused to drink, a second manual assist was performed 5 minutes after the first attempt. If a calf refused to drink the whole contents of the bottle after the second manual assist, milk orts were weighed (Mettler PJ 4000, Mettler Instrument Corp., Hightstown, New Jersey) and recorded. Calves were weaned on day 56 and had *ad libitum* access to water and calf starter until harvested starting on day 77.

3.2.2 *Experimental Design*

This study was divided in two different enrollment dates of August 2020 and January 2021 dairy-beef crossbred calves, respectively, to determine the effectiveness of a capsule containing a 5x10^{9} CFU of *Megasphaera elsdenii* NCIMB 41125 (Lactipro FLX Calf, MS Biotec, Wamego, KS). Regardless of calf enrollment date, the study was conducted in an identical manner in a climate controlled environment to avoid any variations caused by management changes. The study consisted of a 76-day experimental period starting when calves arrived at the University of Kentucky Large Animal Unit. To investigate the effects of the *Megasphaera elsdenii* capsule over time, the 76-day experimental period was divided into 4 main periods of interest: preweaning (day 1 to 41),

weaning (day 42 to 55), postweaning (day 56 to 76), and whole experimental period (day 1 to 76). Upon arrival (day 0), calves had their body weight recorded using an electronic scale (Brecknell PS1000, Avery Weigh-Tronix LLC, Fairmont, MN), and had a 10 mL blood sample collected via jugular venipuncture. Following collection, the jugular blood sample was centrifuged at 3,000 RPM for 15 minutes and the supernatant serum BRIX was measured using a digital refractometer (MISCO PA202, MISCO Solon, OH). Then, calves were assigned to one of three probiotic treatments: placebo capsules on days 15 and 39 (ME_0), probiotic capsule on study day 15 and placebo capsule on day 39 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}). Probiotic and placebo capsules were identical gelatin capsules measuring 35 x 13 mm. However, placebo capsules suffered a dry heat treatment to sterilize the bolus and eliminate active bacteria. All capsules were orally administered using three identical balling guns provided by the probiotic manufacturer. Each probiotic treatment group had its assigned balling gun that was sanitized between applications to avoid accidental inoculations or contaminations. Treatment groups were balanced for weight, age, and serum BRIX (Table 3.2). Treatments were assigned by a third-party scientist, thus all the personnel involved in the study were blinded to the probiotic treatments.

3.2.3 *Calf Performance and Feeding Behavior*

Calf starter orts were weighed daily at 0730h, and individual solid feed intake was determined via disappearance. Once weekly, calf starter samples were collected and stored at -20^{o}C until chemical composition analysis. Samples were dried for 48 h at 55°C in a forced-air oven (Tru-Temp, Hotpack Corp., Philadelphia, PA) to determine the dry matter content of the calf starter, which was further used to calculate solid feed DMI by

multiplying the recorded daily solid feed intake by the weekly calf starter dry matter percentage. Samples were ground using a 2-mm screen (Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA) and composited by month for analysis of chemical composition. Samples were sent to a laboratory (Rock River Laboratory Inc., Watertown, WI) for wet chemistry analysis. Samples were analyzed for crude protein (CP; method 990.03; AOAC International 2023), fat (method 2003.05; AOAC International, 2023), neutral detergent fiber (NDF) with heat-stable α-amylase and sodium sulphite (Van Soest et al., 1991), acid detergent fiber (ADF; method 973.18; AOAC International, 2023), starch (α-amylase method; Hall, 2009), ash (method 942.05; AOAC International, 2023), and minerals (method 985.01 and 923.01; AOAC International, 2023).

Calves were assessed daily for clinical signs of bovine respiratory disease (BRD) and diarrhea according to the University of Kentucky Institutional Animal Care and Use Committee guidelines. A single observer evaluated the calves daily prior to feeding to assess for clinical signs of BRD utilizing the Wisconsin Calf Health Scoring Chart developed by McGuirk and Peek (2014). Briefly, calves had their nasal discharge, eye discharge, ear tilt, rectal temperature, and cough status utilizing a 4-point scoring system that varied from 0 (normal) to 3 (severely abnormal). Additionally, fecal scores were evaluated daily utilizing a 4-point system described by Renaud et al. (2020), where a score of zero indicated normal feces and a score of 3 indicated watery feces. Once weekly, calves were weighed and ADG was calculated by subtracting the most recent weight from the previous recorded weight and dividing it by the number of days between weight recordings. Immediately after the weekly body weight assessment, calves had their lungs assessed for consolidation. Lung consolidation was measured by a single observer utilizing a portable

ultrasound (Ibex Pro, E.I. Medical, Loveland, CO) with 1 cm grid-marks, following the methodology described by Dunn et al. (2018). Further details about the portable ultrasound configuration are thoroughly described by Cantor et al. (2022). A calf was classified as having a minor case of BRD when the sum of scores from the Wisconsin Calf Health Scoring Chart was ≥ 6 and lung consolidation was ≥ 3.0 cm^2. Calves diagnosed with a minor BRD case received enrofloxacin subcutaneously (Baytril, Bayer, Leverkusen, Germany; 1 ml/15 kg of body weight) following the herd veterinarian protocol. Calves that presented sum of scores from the Wisconsin Calf Health Scoring Chart ≥ 6 and lung consolidation was ≥ 5.0 cm^2 were considered as having severe BRD. Calves diagnosed with severe BRD were treated with subcutaneous tulathromycin (Draxxin, Zoetis, Florham Park, NJ; 1.2 ml/45 kg of body weight) and intravenous flunixin meglumine (Banamine, Merck Animal Health, Madison, NJ; 0.5 ml/15 kg of body weight).

Feeding behavior was recorded using timer-activated cameras (Moultrie M40i, Moultrie Feeders Birmingham, AL) hung above each individual pen. Cameras were programmed to record still images on 1-minute intervals for 24h on days 13 and 32, during preweaning, day 53, and 67 during the weaning and postweaning periods, respectively. The 1-minute recording intervals for feeding behavior has been previously validated by Miller-Cushon and DeVries (2011). Eating, drinking, and non-nutritive oral behaviors were recorded by a single observer following a preconstructed ethogram, provided in Table 3.3. Recorded behaviors were summed by day and then summarized by period for statistical analysis.

3.2.4 *Statistical Analysis*

All statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC). All data were checked for normality utilizing the UNIVARIATE procedure and probability distribution plots. We analyzed the overall performance of calves for the whole experimental period as well their performance during the other three periods of interest: preweaning (day 1 to 41), weaning (day 42 to 55), and postweaning (day 56 to 76).

The effects of treatment (e.g. administration of a *Megasphaera elsdenii* capsule) on solid feed DMI, performance, and feeding behavior patterns (time spent eating, drinking, or performing non-nutritive oral behaviors) were determined by analysis of variance using a generalized mixed linear model (Proc MIXED) in SAS. The fixed effects in the model included treatment, enrollment weight, enrollment age, enrollment brix, antibiotic treatment, study period, and the interaction between treatment and period. Study day, week, and period were specified as a repeated measure to analyze solid feed DMI, ADG, and behaviors, respectively. Calf was specified as subject and a compound-symmetry structure was used. Study enrollment date was considered a random factor. Manual stepwise backward elimination retained predictors with a $P < 0.30$. Two calves that received antibiotic treatment for severe BRD (ME_0 n = 1; ME_{15} n = 1) were removed from the analysis. Significant interactions of treatment and period were explored using the SLICE option of the LSMEANS statement of MIXED procedure for each period. Post-hoc comparisons between treatments throughout the study and at each period of interest were conducted utilizing the MULTTEST procedure and the *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979). Significance was declared at $P \leq 0.05$, and

trends were defined as $0.05 < P \leq 0.10$. Authors were unblinded regarding the probiotic treatments as the statistical analysis was finalized to allow for data interpretation.

3.3 RESULTS

Mean solid feed DMI and treatment differences during the preweaning, weaning, postweaning, and whole experimental periods are reported in Table 3.4. In addition, a graphical representation of the mean daily solid feed DMI by probiotic treatment is presented in Figure 3.2. An effect of probiotic treatment on solid feed DMI was observed throughout the whole experimental period, where ME_{15} and ME_{15+39} calves had greater solid feed DMI compared to ME_0 calves ($F_{2,25} = 5.10$; $P = 0.01$; Figure 3.1 - A). No differences between ME_{15} and ME_{15+39} were detected when evaluating solid feed DMI through the whole experimental period (Figure 3.1 - A). Furthermore, we observed a period by treatment interaction ($F_{4,52} = 29.61$; $P < 0.01$; Table 3.4). During the preweaning period, no differences between treatments were found regarding solid feed DMI (Table 3.4). However, ME_{15+39} calves had greater solid feed DMI compared to ME_0 calves during the weaning period (Table 3.4). In addition, ME_{15} calves tended to have greater solid feed DMI compared to ME_0 calves during the weaning period (Table 3.4). No differences between ME_{15} and ME_{15+39} calves were detected when evaluating solid feed DMI during the weaning period (Table 3.4). During the postweaning period, ME_{15} and ME_{15+39} calves had greater solid feed DMI compared to ME_0 calves (Table 3.4). Yet, no differences were observed for solid feed DMI between ME_{15} and ME_{15+39} calves during the postweaning period (Table 3.4).

Mean ADG and treatments differences during the preweaning, weaning, postweaning, and whole experimental periods are reported in Table 3.4. Briefly, treatment had an effect on calves' performance throughout the whole experimental period. We observed an effect of probiotic treatment on ADG ($F_{2,25} = 5.17$; $P = 0.01$; Figure 3.1 - B), where ME_{15} and ME_{15+39} calves had greater ADG compared to ME_0 calves (Table 3.4). No differences between ME_{15} and ME_{15+39} were detected when evaluating ADG through the whole experimental period (Figure 3.1 - B). Furthermore, we observed a period by treatment interaction ($F_{4,52} = 3.41$; $P = 0.02$; Table 3.4). During the preweaning period, no differences between treatments were found (Table 3.4). However, ME_{15} calves had greater ADG when compared to ME_0 calves during the weaning period (Table 3.4). In addition, ME_{15+39} calves tended to have greater ADG compared to ME_0 calves during the weaning period (Table 3.4). No differences between ME_{15} and ME_{15+39} calves were detected when evaluating ADG during the weaning period (Table 3.4). During the postweaning period, ME_{15} and ME_{15+39} calves had greater ADG compared to ME_0 calves (Table 3.4). Yet, no differences were observed for ADG between ME_{15} and ME_{15+39} calves during the postweaning period (Table 3.4).

Mean time spent eating, drinking water, and performing non-nutritive oral behaviors and treatment differences during the preweaning, weaning, postweaning, and whole experimental periods are reported in Table 3.5. Briefly, we did not observe an effect of treatment ($F_{2,25} = 0.47$, $P = 0.63$; Table 3.5) nor a treatment by period interaction ($F_{4,52} = 1.00$, $P = 0.42$; Table 3.5) for time spent eating. We found an effect of probiotic treatment on the time calves spent drinking water ($F_{2,25} = 5.70$, $P < 0.01$; Table 3.5) throughout the whole experimental period, where ME_{15} and ME_{15+39} calves spent more time drinking

water compared to ME_0 calves (Table 3.5). No difference between ME_{15} and ME_{15+39} was detected regarding time spent drinking water when evaluating the whole experimental period (Table 3.5). Furthermore, we observed a period by treatment interaction ($F_{4,52}$ = 3.43; P = 0.01; Table 3.5) regarding the time calves spent drinking water. During the preweaning period, the time calves spent drinking water did not differ between treatments (Table 3.5). However, ME_{15+39} calves spent more time drinking water compared to ME_0 calves during the weaning period (Table 3.5). In addition, ME_{15} calves tended to spend more time drinking water compared to ME_0 calves during the weaning period (Table 3.5). No difference between ME_{15} and ME_{15+39} calves was detected when evaluating time spent drinking water during the weaning period (Table 3.5). During the postweaning period, ME_{15} calves spent more time drinking water compared to ME_0 calves (Table 3.5). No differences in time spent drinking water during the post weaning period were observed between ME_{15+39} and ME_0, nor between ME_{15} and ME_{15+39} calves (Table 3.5). The effects of the *Megasphaera elsdenii* capsule throughout the whole experimental period tended to affect the time calves spent performing non-nutritive oral behaviors ($F_{2,26}$ = 2.69, P = 0.09; Table 3.5), yet no differences between treatments were found (Table 3.5). No treatment by period interaction was detected regarding the time calves spent performing non-nutritive oral behaviors ($F_{4,52}$ = 0.65, P = 0.63; Table 3.5).

3.4 DISCUSSION

In this study we evaluated the effectiveness of an oral probiotic capsule containing *Megasphaera elsdenii* in dairy-beef crossbred calves. The results from this study indicate that there might be benefits to utilizing a *Megasphaera elsdenii* capsule in dairy-beef

crossbred calves during the preweaning period, with improvements in solid feed DMI and performance. This study builds upon previous work showing possible benefits of oral interventions with *Megasphaera elsdenii* on Holstein cows (Mazon et al., 2020) and calves (Muya et al., 2015). However, the effects of oral interventions with *Megasphaera elsdenii* on solid feed DMI, performance, and feeding behavior patterns of dairy-beef crossbred calves have not been previously investigated.

We found that the strategic intervention with *Megasphaera elsdenii* probiotic capsule improved overall solid feed DMI in dairy-beef crossbred calves. Although no differences between treatments were seen during the preweaning period, calves receiving *Megasphaera elsdenii* showed greater solid feed DMI during the weaning and postweaning periods. Other authors have reported similar preweaning solid feed DMI to dairy calves under similar milk feeding programs (Khan et al., 2007a, Mirzaei et al., 2020). Furthermore, our results agree with previous studies conducted with Holstein calves, lambs, and beef steers. First, Muya et al. (2015) also reported that Holstein calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age had greater solid feed DMI compared to control calves. However, the same authors reported that the increase in solid feed DMI started during the preweaning period, which was not observed in the present study. We believe that this time difference regarding solid feed DMI might be associated with the fact that in this study the calves were in a higher allowance of milk compared to Muya et al. (2015). In another trial with Holstein calves receiving a *Megasphaera elsdenii* drench at 14 days of age, Muya et al. (2017) presented greater solid feed DMI during the preweaning and postweaning periods compared to control calves. In addition, Henning et al. (2010a) reported that lambs and steers receiving a *Megasphaera elsdenii* drench one

day prior to transitioning from a high-forage to a high-grain diet also presented greater DMI following drench administration compared to animals receiving a placebo drench. On the other hand, Yohe et al. (2018) provided a *Megasphaera elsdenii* drench to Holstein heifers at 14 days of age and did not observe any effects of probiotic treatment on solid feed DMI between treatments. However, these authors had a limited sample size and did not power their study with the objective of detecting differences in solid feed DMI. We hypothesize that the probiotic intervention might have affected reticulorumen pH and VFA dynamics by favoring the reticulorumen development and, consequently, solid feed DMI in ME_{15} and ME_{15+39} calves. Previous researchers have reported a positive association between solid feed DMI, reticulorumen development, and VFA absorption capacity in calves (Baldwin et al., 2004, Khan et al., 2011b, Nishihara et al., 2023). Hence, when the reticulorumen is unable to absorb the VFA produced due to poor development, drastic drops in reticulorumen pH and subsequent damage of the reticulorumen epithelium can be observed, resulting in loss of appetite (Plaizier et al., 2008, Li et al., 2019). Future research should investigate the effects of the *Megasphaera elsdenii* probiotic on reticulorumen pH, VFA dynamics, and development of calves.

Performance was also improved by the probiotic treatment, probably a consequence of the increased solid feed DMI. We found that the strategic intervention with *Megasphaera elsdenii* probiotic capsule also improved overall ADG in dairy-beef crossbred calves. Following solid feed DMI, no differences in ADG between treatments were seen during the preweaning period. However, calves receiving *Megasphaera elsdenii* showed greater ADG during the weaning and postweaning periods. Other authors have reported similar ADG both for dairy (Mirzaei et al., 2020) and dairy-beef crossbred calves

(Hickson et al., 2014) reared under similar conditions to the present study. Furthermore, our results agree with previous studies conducted with Holstein calves and beef steers. First, Muya et al. (2017) reported that dairy calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age had greater ADG during the postweaning period compared to control calves. In addition, Muya et al. (2015) reported that Holstein calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age tended to have greater ADG during the whole experimental period compared to control calves. Henning et al. (2010a) also reported that beef steers receiving a *Megasphaera elsdenii* drench prior to transitioning from a high-forage to a high-grain diet presented greater ADG compared to animals receiving a placebo drench. Our results contrast with the findings reported by Yohe et al. (2018), as the authors did not observe any differences in calf growth between treatments when providing a *Megasphaera elsdenii* drench to Holstein heifers at 14 days of age. However, as previously discussed, these authors did not design their study with the objective of detecting differences in animal performance.

Although we observed that calves ME_{15} and ME_{15+39} calves had greater solid feed DMI than ME_0 calves, strategic intervention with *Megasphaera elsdenii* probiotic capsule did not affect the time that calves spent eating but did positively affect time spent drinking water compared to placebo. Furthermore, we did not observe any differences between treatments regarding the expression of non-nutritive oral behaviors. To our knowledge, the present study is the first to look at feeding behavior in dairy-beef crossbred calves supplemented with *Megasphaera elsdenii*. Still, our feeding behavior results for time spent eating, drinking, and performing non-nutritive oral behaviors are within the range reported for dairy calves (Hepola et al., 2008, Castells et al., 2012, Montoro et al., 2013). Supporting

our results about water drinking time, Hepola et al. (2008) concluded that dairy calves spend little time drinking water preweaning, but this time increases towards the weaning and postweaning periods. Kertz et al. (1984) reported high associations between water and solid feed DMI towards the end of the rearing period, which might explain the reason that calves receiving the *Megasphaera elsdenii* capsule spent more time drinking water than calves receiving a placebo capsule. In the present study, calves had access to calf starter and water through buckets, which did not allow us to record water intake, solid feed meal size, meal duration, and solid feed intake rate in real time. Changes in meal size, duration, and feeding rate have been associated with SARA occurrence in dairy cows (Krauze and Oetzel, 2006, DeVries et al., 2009). However, real time monitoring of water and solid feed intake is laborious when not automated and was not part of the main hypotheses in this experiment. Real time monitoring of water and solid feed intake is possible through the use of automated water and solid feed intake recording systems such as the one validated by Chapinal et al. (2007). Thus, future research should further investigate the effects of the *Megasphaera elsdenii* probiotic capsule on the feeding behavior of calves utilizing automated water and solid feed intake recording systems.

Overall, we did not observe differences in solid feed DMI, performance, or behavioral patterns differences between ME_{15} and ME_{15+39} calves. Therefore, it is still unclear whether there are any additional benefits of providing calves with an additional *Megasphaera elsdenii* capsule around the time of weaning. Weaning is a sensitive and challenging time for calves, as the establishment of a consistent and adequate solid feed diet is essential to its success. In the present study, calves were gradually weaned, which allowed them to increase their solid feed DMI prior to total removal of the liquid diet (Khan

et al., 2007a, Sweeney et al., 2010). Yet, the transition from liquid to solid diets can be challenging for calves. During weaning, calves display an increase in vocalizations (Thomas et al., 2001) and expression of non-nutritive oral behaviors (Jensen, 2003), which might be associated with hunger, a major welfare challenge in commercially raised calves (see review by (Costa et al., 2019). Furthermore, during the weaning period, the calf experiences drastic physical, metabolic, and microbiome changes triggered by changes in the diet (Baldwin et al., 2004, Kim et al., 2016). In a recent review, Cangiano et al. (2020) suggested that utilizing microbial-based products around weaning might prevent calves from experiencing negative health-related events that affect growth during the weaning and postweaning periods. Thus, the timing of *Megasphaera elsdenii* intervention and its effects on reticulorumen pH and VFA dynamics should be further investigated. Our results showed that the providing calves with a *Megasphaera elsdenii* capsule during the preweaning period has the potential to be used as a strategic management tool, especially in preparation for the weaning and postweaning periods. In finishing beef cattle, *Megasphaera elsdenii* has been used strategically to alleviate aversive effects associated with accelerated dietary step-up protocols (DeClerck et al., 2020a). With the availability of automated calf starter feeders, farmers can utilize solid feed intake data to identify calves with abnormal solid feed intake and make management decisions, such as adjusting calf weaning age (de Passillé and Rushen, 2012, Benetton et al., 2019) or potentially by intervening with a probiotic such as *Megasphaera elsdenii*. In this study, we opted to use an autoclaved *Megasphaera elsdenii* probiotic capsule as a placebo as we did not expect any postbiotic effects of the microorganism. Postbiotics are non-living microorganisms or microorganism components that might confer a health benefit to the host (Salminen et al., 2021). To our

knowledge, there is no current literature indicating that *Megasphaera elsdenii* is being used nor acts as postbiotic. Future research should focus on the strategic uses of the *Megasphaera elsdenii* probiotic capsule and investigate how administration time might affect reticulorumen pH, VFA dynamics, and development.

3.5 CONCLUSION

The results from this study indicate that there might be benefits to utilizing a *Megasphaera elsdenii* capsule in dairy-beef crossbred calves. We found that calves receiving the *Megasphaera elsdenii* capsule had greater solid feed DMI and ADG compared to calves receiving a placebo capsule. Furthermore, calves receiving the *Megasphaera elsdenii* capsule spent more time drinking water than placebo calves. It is still unclear if there are benefits of providing two doses of the probiotic capsule to the calves. Future research should investigate the effects of the *Megasphaera elsdenii* capsule on the reticulorumen pH, VFA dynamics, and development of dairy-beef crossbred calves.

Table 3.1 The Mean ± (SD) chemical composition of the pelletized calf starter (Special Calf Starter and Grower, Bagdad Roller Mills Inc, Baghdad, KY) offered to dairy-beef crossbred calves (n = 31) assigned to different probiotic treatments containing *Megasphaera elsdenii*.

Variables Component[1]	Mean ± SD
DM, %	90.14 ± 1.58
CP	21.70 ± 1.04
Fat	2.78 ± 0.20
NDF	10.90 ± 0.65
ADF	4.34 ± 0.35
Starch	38.29 ± 2.72
Ash	7.69 ± 0.64
Calcium	1.20 ± 0.09
Phosphorus	0.57 ± 0.04
Magnesium	0.20 ± 0.01
Potassium	1.18 ± 0.09
Sulfur	0.27 ± 0.02
ME (Mcal/kg)[1]	2.12 ± 0.03

[1]Expressed in % DM unless specified otherwise.

[2] ME = TDN × 0.04409 × 0.82; calculated according to (NRC, 2001)

Table 3.2 Mean, minimum, and maximum body weight (kg), age (d), and BRIX (%) at study enrollment for dairy-beef crossbred (n = 31). Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study.

	Probiotic Treatment								
	ME_0 (n = 10)			**ME_{15}** (n = 10)			**ME_{15+39}** (n = 11)		
	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum
Body weight, kg	46.34 ± 8.33	33.20	65.40	45.56 ± 6.94	37.40	59.60	44.15 ± 6.40	34.40	55.00
Age, days	7.80 ± 2.35	4.00	12.00	8.30 ± 1.83	6.00	12.00	8.36 ± 2.01	6.00	12.00
BRIX, %	8.45 ± 0.62	7.70	9.80	8.26 ± 0.56	7.40	9.40	8.23 ± 0.68	7.10	9.40

Table 3.3 Ethogram of behaviors used to record time spent eating, time spent drinking water, and time spent performing non-nutritive oral behaviors from dairy-beef crossbred calves (n = 31) assigned to different probiotic treatments containing *Megasphaera elsdenii*.

Behavior	Definition
Eating	The calf has its muzzle placed below the calf starter bucket's rim
Drinking	The calf has its muzzle placed below the water bucket's rim
Non-nutritive oral behaviors	The calf has his mouth in direct contact or less than 30 cm of the bucket holder, the bucket's handle, the bucket's rim, the carabiner clip that holds the bucket, or the wall directly above the bucket holder

Table 3.4 Least squares mean ±SEM of daily solid feed dry matter intake (DMI; kg/d) and average daily gain (ADG; kg/d) for dairy-beef crossbred calves (n = 31). Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study. Results are shown separately for the preweaning, weaning, and postweaning periods.

	Probiotic Treatment				*P*-value[1,2]			
Item	ME_0	ME_{15}	ME_{15+39}	SEM	Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Preweaning (d 1 to 41)								
Solid Feed DMI (kg/d)	0.08	0.15	0.26	0.13	0.57	NS	NS	NS
ADG (kg/d)	0.60	0.69	0.74	0.09	0.52	NS	NS	NS
Weaning (d 42 to 55)								
Solid Feed DMI (kg)	0.95	1.43	1.50	0.14	0.01	0.08	0.03	NS
ADG (kg/d)	0.57	1.02	0.93	0.10	0.01	0.02	0.07	NS
Postweaning (d 56 to 76)								
Solid Feed DMI (kg)	2.49	3.28	3.29	0.13	<0.01	<0.01	<0.01	NS
ADG (kg/d)	1.13	1.58	1.58	0.10	<0.01	0.02	0.01	NS

[1] *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979)

[2] NS represents a Bonferroni-adjusted *P*-value ≥ 1.0

Table 3.5 Least squares mean ±SEM of time spent eating (min/d), time spent drinking(min/d), and time spent performing non-nutritive oral behaviors (min/d) for dairy-beef crossbred calves (n = 31). Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study. Results are shown separately for the preweaning, weaning, postweaning, and whole experimental period.

	Probiotic Treatment				*P*-value[1,2]			
Item	ME_0	ME_{15}	ME_{15+39}	SEM	Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Preweaning (d 1 to 41)								
Eating (min/d)	22.29	16.73	19.72	5.44	0.67	NS	NS	NS
Drinking water (min/d)	1.71	2.15	4.11	1.40	0.39	NS	NS	NS
Non-nutritive oral behaviors (min/d)	26.52	28.11	22.97	5.48	0.64	NS	NS	NS
Weaning (d 42 to 55)								
Eating (min/d)	39.18	47.56	46.08	6.47	0.54	NS	NS	NS
Drinking water (min/d)	1.68	8.04	8.43	1.68	0.01	0.07	0.04	NS
Non-nutritive oral behaviors (min/d)	42.19	37.11	25.28	6.49	0.08	NS	0.26	NS
Postweaning (d 56 to 76)								
Eating (min/d)	68.63	69.00	77.91	6.47	0.39	NS	NS	NS
Drinking water (min/d)	4.69	12.93	10.25	1.68	< 0.01	0.01	0.11	NS
Non-nutritive oral behaviors (min/d)	32.27	32.78	25.64	6.49	0.35	NS	NS	NS

Table 3.5 Continued

Item	Probiotic Treatment			SEM	*P*-value[1,2]			
	ME_0	ME_{15}	ME_{15+39}		Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Whole experimental period (d 1 to 76)								
Eating (min/d)	43.37	44.43	47.91	4.81	0.63	NS	NS	NS
Drinking water (min/d)	2.69	7.71	7.60	1.22	0.01	0.02	0.02	0.95
Non-nutritive oral behaviors (min/d)	34.99	32.67	24.63	4.82	0.09	0.63	0.11	0.21

[1] *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979)

[2] NS represents a Bonferroni-adjusted *P*-value ≥ 1.0

Figure 3.1 Differences in daily solid feed dry matter intake (A; kg/d) and average daily gain (B; kg/d) during the 76-day experimental period expressed as least square means ± SEM in dairy-beef crossbred calves (n = 31). Calves were assigned to one of three probiotic treatments containing Megasphaera elsdenii: placebo (ME0; grey), probiotic capsule on study day 15 (ME15; green), or probiotic capsule on days 15 and 39 (ME15+39; blue) of the study.

[a-b] Different superscripts indicate significant differences between probiotic treatments ($P < 0.05$)

Figure 3.1 A)

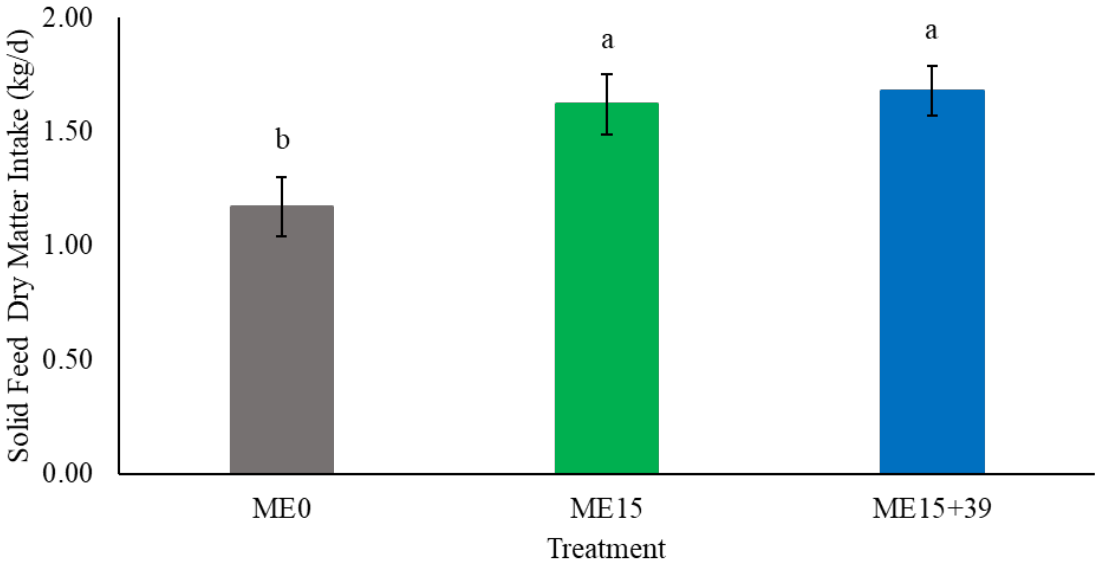


Figure 3.1 B)

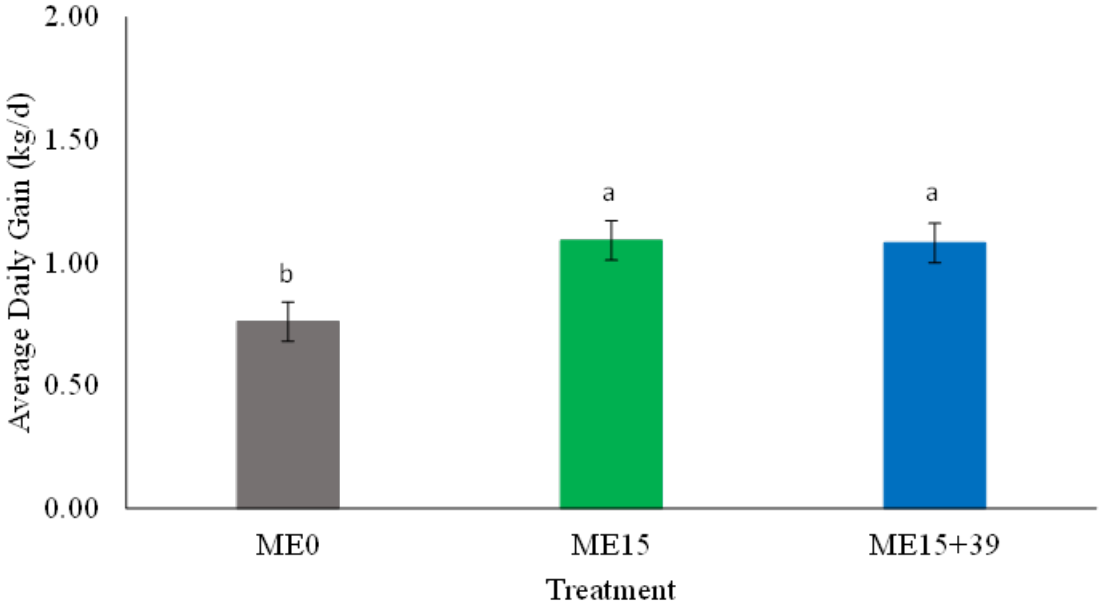
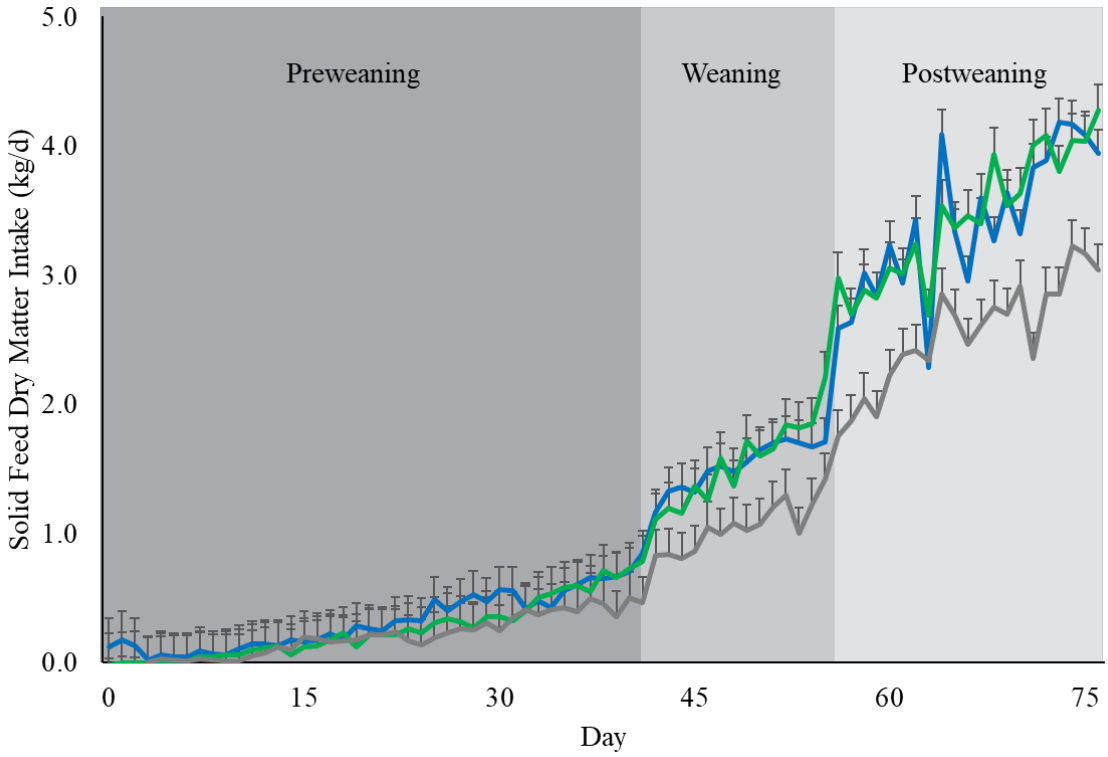

2.00
1.50
1.00
0.50
0.00
Average Daily Gain (kg/d)
b
a
a
ME0
ME15
ME15+39
Treatment

Figure 3.2 Daily solid feed dry matter intake (kg/d) expressed as least square means ± SEM in dairy-beef crossbred calves (n = 31). Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME0; grey), probiotic capsule on study day 15 (ME15; green), or probiotic capsule on days 15 and 39 (ME15+39; blue) of the study.



CHAPTER 4. EFFECTS OF A *MEGASPHAERA ELSDENII* ORAL CAPSULE ON THE DEVELOPMENT OF DAIRY-BEEF CROSSBRED CALVES. PART II: RETICULORUMEN PH, VFA, AND ANATOMICAL DEVELOPMENT

4.1 INTRODUCTION

Many disorders can affect a calf's reticulorumen environment and have negative consequences for animal growth and development. One of these disorders is subacute ruminal acidosis (SARA), characterized by low reticulorumen pH that can affect reticulorumen tissue, animal physiology, feed intake, milk yield, and milk composition (Plaizier et al., 2008, Plaizier et al., 2022). Previous studies have reported that reticulorumen pH in calves seems to be consistently lower than in adult cattle (Gentile et al., 2004, Suarez-Mena et al., 2016, Yohe et al., 2018). While deeply studied in cattle, there is still limited research on the occurrence and impacts of SARA in calves.

Solid feed intake is fundamental for the development and growth of the reticulorumen in calves (Baldwin et al., 2004, Khan et al., 2011b). However, calves are susceptible to decreases in reticulorumen pH during weaning as they transition from liquid to solid diets (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020). Recent studies have reported that calves with calf starter induced SARA have displayed reduced solid feed dry matter intake and body weights, starting as early as four weeks of age (Li et al., 2019, Gelsinger et al., 2020). In addition to the negative effects on intake and performance, SARA might affect reticulorumen volatile fatty acids (VFA) and anatomical development in calves. Gelsinger et al. (2020) reported that calves with calf starter induced SARA had greater concentrations of total VFA, as well as greater concentrations of propionate and isobutyrate compared to calves fed a control calf starter. The same authors

reported that calves with calf starter induced SARA tended to have a lesser reticulorumen weight and displayed greater reticulorumen lesion scores compared with calves fed a calf starter designed to blunt SARA.

In mature cows under induced SARA, Steele et al. (2011) reported reductions in the depth of the stratum basale, spinosum, and granulosum layers of the reticulorumen epithelium. In calves, Li et al. (2019) reported that SARA can also affect biological pathways associated with cell division, possibly affecting reticulorumen epithelial health and anatomical development. Damages to the reticulorumen epithelium have been associated with the incidence of liver abscesses, as they might allow microorganisms to enter the bloodstream (Nagaraja and Titgemeyer, 2007a, Elmhadi et al., 2022). Lastly, long exposure to SARA can lead to metabolic acidosis and death in calves (Gentile et al., 2004).

The production and absorption of VFA provides the chemical stimulus needed for the development of the reticulorumen epithelium in calves (Sander et al., 1959, Tamate et al., 1962, Diao et al., 2019). However, diets with elevated proportions of non-fibrous carbohydrates might result in VFA accumulation and lactate production causing rapid pH declines in the reticulorumen (Laarman and Oba, 2011). In a study investigating the pre to postweaning papillae growth and reticulorumen fermentation characteristics in calves, van Niekerk et al. (2021) reported that greater reticulorumen papillae development might aid VFA absorption, reducing SARA time in calves. Furthermore, Nishihara et al. (2023) reported a positive association between reticulorumen papillae dimensions and genes associated with VFA absorption and ketogenic activity. Thus, mitigation strategies that promote reticulorumen development and reduce the negative effects of SARA in preweaning calves are warranted.

There is a myriad of SARA prevention methods in adult cattle. One of them involves dietary supplementation with probiotics containing lactate-utilizing bacteria, such as *Megasphaera elsdenii* (*Megasphaera elsdenii*) (Nocek et al., 2002). *Megasphaera elsdenii* is a naturally occurring rumen microorganism known for utilizing lactate and stabilizing ruminal pH (Counotte et al., 1981b, Nagaraja and Titgemeyer, 2007c, Susanto et al., 2023). In a recent study with mid-lactation cows under a SARA challenge, Mazon et al. (2020) reported that challenged cows receiving a probiotic containing *Megasphaera elsdenii* displayed greater reticulorumen pH and experienced shorter and milder SARA compared to control cows. As such, there appears to be an opportunity to implement strategic probiotic interventions in calves. For example, Cangiano et al. (2020) suggested that the strategic supplementation of microbial-based products around weaning might mitigate the reticulorumen exposure to conditions that can have a negatively impact the health and performance on calves. However, the current literature investigating the effects of *Megasphaera elsdenii* on calf reticulorumen pH, VFA, and anatomical development is limited. Thus, the objective of this study was to evaluate the effects of an oral probiotic capsule containing a live culture of *Megasphaera elsdenii* NCIMB 41125 (Lactipro FLX Calf, MS Biotec) on the reticulorumen pH, VFA, and anatomical development of dairy-beef crossbred calves.

4.2 MATERIALS AND METHODS

4.2.1 *Animals and Experimental Design*

This study was conducted between August 2020 and April 2021 under Institutional Animal Care and Use Committee Protocol number 2019-3156 at the University of

Kentucky Large Animal Unit. This study is described in greater detail in the companion to this paper (Mazon et al., 2023). Briefly, dairy-beef calves (Holstein x Angus; n = 31; 45.3 ± 7.1kg; 8.2 ± 2.0 d old) were enrolled in a 76-day randomized trial in 2 enrollment dates (August 2020 and January 2021) to determine the effectiveness of a capsule containing a $5x10^9$ CFU of *Megasphaera elsdenii* NCIMB 41125 (Lactipro FLX Calf, MS Biotec, Wamego, KS). Calves were assigned to one of three probiotic treatments balanced for weight, age, and passive transfer status: an autoclaved probiotic capsule as placebo (ME_0), a single dose of probiotic on day 15 (ME_{15}), or probiotic administration on days 15 and 39 (ME_{15+39}). Treatments were assigned by a third-party researcher, hence all the personnel involved in the study were blinded to the probiotic treatments. Calves were fed 7 L/d of milk replacer divided in two equal meals (Cow's Match Warm Front, Land O Lakes, MN; 150 g of replacer for 1L of water heated to 40ºC; 13.04% DM; 27% CP; 10% crude fat, and 40% lactose) until day 41. On day 42, milk allowance per meal was reduced in half until weaning on day 56. Calves were individually housed and had unlimited access to water and pelletized calf starter (21.70 % CP, 2.78% fat; 38.29% starch) throughout the study. Calves were assessed daily for signs of bovine respiratory disease (McGuirk and Peek, 2014) and diarrhea (Renaud et al., 2020). Calf starter intake was recorded daily via disappearance. Once weekly, calves were weighed had their lung consolidation measured following Dunn et al. (2018). Further information on the animals, housing, diets, health evaluation, and disease treatment protocols utilized in this study are included in the companion paper (Mazon et al., 2023).

4.2.2 *Reticulorumen pH and VFA*

Reticulorumen fluid was collected from each calf on days 14, 35, 49, 58, and 70 starting at 4h post-feeding. Reticulorumen fluid was collected with an esophageal tube following the stomach tubing procedures described by Terré et al. (2013a). Briefly, a flexible esophageal tube connected to a Geishauser probe (Geishauser and Gitzel, 1996) was inserted into the reticulorumen via the esophagus. The esophageal tube was connected to a sterile 1200 mL suction canister (Medi-Vac Guardian, Cardinal Health, Waukegan, IL), which was connected to an electric variable vacuum pump (Gen-Med EA, General Medical Corporation, Richmond, VA). After the esophageal tube was inserted, researchers started the vacuum pump. First, samples with any visual signs of saliva contamination were discarded. A reticulorumen fluid sample was considered contaminated with saliva if the researchers detected any visual signs of viscous and slight transparent fluid in the sample. After all saliva was discarded, a new sterile suction canister was utilized to collect the reticulorumen fluid sample. To avoid any possible carryover between samples, the esophageal tube and Geishauser probe were washed with warm water, sanitized using a 0.05% chlorhexidine solution (Freeman and Auer, 2012), and rinsed with nanopure water prior to reutilization. Reticulorumen fluid samples were filtered through four layers of cheesecloth. A subsample was used for pH recording using an electronic meter (S220, Mettler Toledo, Switzerland). The remaining fluid was stored at -80ºC until VFA analysis.

Reticulorumen VFA (acetate, propionate, and butyrate) concentrations were determined via gas chromatography (Erwin et al., 1961). Briefly, in a 2 mL microcentrifuge tube, 1 mL of reticulorumen fluid was mixed with 0.1 mL of a 50% solution of metaphosphoric acid and 0.1 mL of 2-ethyl butyrate (85mM). Samples were

then centrifuged at 39,000 x g, at 5 °C for 20 min. The supernatant fluid was transferred into 2mL autosampler vials and analyzed using a gas chromatograph in duplicates (Agilent Technologies, Inc., Santa Clara, CA). Following VFA analysis, total VFA concentration was calculated by summing the concentration of each individual VFA. Also, acetate to propionate ratio was calculated by dividing the obtained concentration of acetate by the propionate concentration.

4.2.3 *Calf Dissections*

Starting on day 77 of the study, calves (86.2 ± 2.2 days old) were euthanized and dissected for reticulorumen anatomical development, pH, and VFA profile assessment. Calves were stunned using a captive bolt and exsanguinated after stunning. Following exsanguination, the digestive tract was assessed by performing an incision that ranged from the sternum to the anus. Prior to the digestive tract removal, the esophagus and rectum were closed using cable ties. The digestive tract was then removed and placed in a plastic tray. Following Steele et al. (2017), cable ties were placed on the reticulo-omasal and omaso-abomasal orifices to allow separation between the reticulorumen and omasum and between the omasum and abomasum respectively. Reticulorumen, omasum, and abomasum were first weighed full. Then each compartment was emptied, rinsed with cold water, and weighed empty. Prior to emptying, reticulorumen fluid samples were taken for pH and VFA analysis using the previously described methodology. In addition, researchers recorded the weight of each animal's liver, kidneys, and spleen.

Following Lesmeister et al. (2004), a reticulorumen tissue sample (approximately 5 cm^2) was collected from the ventral sac for analysis of papillae length, width, and histomorphometric analysis. Tissue samples were thoroughly rinsed with phosphate

buffered saline and stored 50 ml tubes containing formalin for 48 h. After 48 h, the formalin was replaced with 70 % ethanol. Tissue samples were kept in a low-light environment at room temperature until further processing for microscopy.

To assess papillae length, width, and area, researchers dissected 20 individual papillae per calf and individual papillae images were captured using a stereo microscope (Leica EZ4W, Leica Microsystems, Milton Keynes, UK) at 8 x magnification following van Niekerk et al. (2021). Papillae length, width, and area were assessed utilizing an image analysis software (ImageJ, National Institutes of Health, Bethesda, MD). Papillae length was measured starting at the middle point perpendicular to the papillae base. Papillae width was measured at the median point of the papillae. Papillae area was calculated by outlining the whole papillae and the result was multiplied by two to obtain the two-sided surface area (van Niekerk et al., 2021).

Reticulorumen tissue was prepared for light microscopy following Odongo et al. (2006) and Steele et al. (2011). Briefly, tissue samples were dehydrated, submerged in paraffin wax, sectioned in 4-µm-thick slices, and stained with hematoxylin and eosin (Steele et al., 2011). Five papillae per calf were randomly selected and individual papillae images were captured using a light microscope (AXIO Zoom. V16, ZEISS, Oberkochen, Germany) at 11.5 x magnification (2.3 x objective combined with 5 x zoom). The thickness of the epithelial layers was assessed at four randomly selected sites per papillae utilizing an image analysis software (ImageJ, National Institutes of Health, Bethesda, MD). Reticulorumen epithelial layers were defined according to Steele et al. (2011). Stratum spinosum and basale thickness were measured as one, defined as the cluster of cells between the lamina propria and the stratum granulosum. Stratum granulosum thickness

was defined as the layer of cells displaying long axis laying perpendicular to the stratum spinosum and basale. Lastly, stratum corneum was defined as the heavily stained layer composed of cell fragments (Steele et al., 2011). Researchers were blinded regarding the probiotic treatments throughout the whole papillae dissection and measurement process.

4.2.4 *Statistical Analysis*

Statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC). Data were assessed for normality utilizing the UNIVARIATE procedure and probability distribution plots and no outliers were identified. We analyzed the reticulorumen pH and VFA concentration for the whole experimental period and by day.

The effects of the *Megasphaera elsdenii* capsule on reticulorumen pH and VFA concentration for the whole experimental period and by day were determined using a mixed linear model (PROC MIXED). The model included probiotic treatment, enrollment weight, enrollment age, enrollment brix, calf starter dry matter intake, antibiotic treatment, sampling day, and the interaction between treatment and day. Day was specified as a repeated measure and calf as subject, using a compound-symmetry structure. Study enrollment date was considered a random factor. Manual stepwise backward elimination retained predictors with a $P < 0.30$. Significant interactions of probiotic treatment and day were explored using the SLICE option of the LSMEANS statement of MIXED procedure for each day. Post-hoc comparisons between treatments throughout the study and at each day of interest were conducted utilizing the MULTTEST procedure and the *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979).

The effects of the *Megasphaera elsdenii* capsule on reticulorumen pH, reticulorumen VFA concentration, forestomach weights, visceral organ weights, and papillae

development (height, width, area, and epithelial layers) at dissection were determined using a mixed linear model (PROC MIXED). The model included probiotic treatment, enrollment weight, enrollment age, enrollment brix, and antibiotic treatment. Study enrollment date was considered a random factor. Post-hoc comparison between treatments were determined utilizing the PDIFF option in SAS and adjusted using the Bonferroni method (Holm, 1979).

Calves that received extended antibiotic treatment for severe bovine respiratory disease (n = 2) were removed from all analyses. Significance was declared at $P \leq 0.05$, and trends were defined as $0.05 < P \leq 0.10$. After data analysis was completed, authors were unblinded regarding the probiotic treatments to allow for data interpretation.

4.3 RESULTS

Mean reticulorumen pH, total VFA, acetate concentration, propionate concentration, butyrate concentration, and acetate to propionate ratio and treatment differences over the whole experimental period are presented in Table 4.1. Briefly, probiotic treatment tended to reduce reticulorumen pH ($F_{2,26} = 2.49$; $P = 0.10$; Table 4.1), yet no differences between individual probiotic treatments were detected throughout the whole experimental period (Table 4.1). In addition, no probiotic treatment by day interaction was seen regarding reticulorumen pH ($F_{12,97} = 0.61$; $P = 0.83$). Probiotic treatment tended to affect total VFA ($F_{2,25} = 2.96$; $P = 0.07$; Table 4.1) over the whole experimental period as ME_{15+39} tended to have greater total VFA when compared to ME_0 (Table 4.1). No differences between ME_{15} and ME_{15+39} nor between ME_{15} and ME_0 were seen regarding total VFA over the whole experimental period. In addition, no treatment by

day interactions was seen regarding total VFA ($F_{8.98} = 0.83$; $P = 0.58$). Probiotic treatment tended to affect acetate concentration throughout the experimental period ($F_{2,25} = 2.75$; $P = 0.08$; Table 4.1) as ME_{15+39} tended to have greater acetate concentrations compared to ME_0 (Table 4.1). No differences between ME_{15+39} and $ME_{15,}$ nor between ME_0 and ME_{15} were seen (Table 4.1). No treatment by day interactions were seen regarding acetate concentrations ($F_{8,98} = 0.99$; $P = 0.45$).

There was a probiotic treatment effect on propionate concentration over the whole experimental period ($F_{2,24} = 3.88$; $P = 0.04$; Table 4.1). During the whole experimental period, ME_{15+39} calves showed greater propionate concentrations compared to ME_0 calves (Table 4.1). No differences between ME_{15+39} and $ME_{15,}$ or between ME_0 and ME_{15} were seen (Table 4.1). No treatment by day interaction was seen regarding propionate concentrations ($F_{8,98} = 0.68$; $P = 0.71$). No effects of probiotic treatment ($F_{2,26} = 1.06$; $P = 0.36$) or treatment by day interaction ($F_{8,98} = 1.54$; $P = 0.15$) were seen when evaluating reticulorumen butyrate concentrations during the whole experimental period. No probiotic treatment effect was seen on acetate to propionate ratio over the whole experimental period ($F_{2,26} = 0.64$; $P = 0.54$; Table 4.1). Furthermore, no treatment by day interaction was seen regarding acetate to propionate ratio ($F_{8,98} = 1.26$; $P = 0.28$).

4.3.1 Calf Dissection

Mean reticulorumen, omasum, abomasum, and visceral organ weights and treatment differences at dissection are presented in Table 4.2. Briefly, probiotic treatment tended to affect full reticulorumen weight ($F_{2,23} = 3.11$; $P = 0.06$; Table 4.2) as ME_{15+39} tended to have greater reticulorumen weight compared to ME_0. No differences in full rumen weight between ME_{15+39} and $ME_{15,}$ or between ME_0 and ME_{15} were seen (Table 4.2). Furthermore,

probiotic treatment affected empty reticulorumen weight ($F_{2,23} = 4.93$; $P = 0.02$; Table 4.2) as ME_{15} calves had greater empty reticulorumen weights than ME_0 and ME_{15+39} calves (Table 4.2). No differences in empty reticulorumen weight were seen between ME_{15+39} and ME_0 calves (Table 4.2). Probiotic treatment did not affect the full or empty weights of the omasum ($F_{2,22} \leq 1.06$; $P \geq 0.36$; Table 4.2) and abomasum ($F_{2,23} \leq 1.54$; $P \geq 0.24$; Table 4.2). Furthermore, probiotic treatment affected liver weight ($F_{2,23} = 4.71$; $P = 0.02$; Table 4.2), as ME_{15} and ME_{15+39} had greater liver weights compared to ME_0 (Table 4.2). No differences in liver weight were seen between ME_{15} and ME_{15+39} calves (Table 4.2). Probiotic treatment did not affect kidney ($F_{2,23} = 0.74$; $P = 0.49$; Table 4.2) or spleen ($F_{2,23} = 0.35$; $P = 0.71$; Table 4.2) weights.

Reticulorumen pH at dissection, reticulorumen VFA concentration at dissection, reticulorumen papillae and strata measurements are presented in Table 4.3. Briefly, probiotic treatment did not affect reticulorumen pH ($F_{2,23} = 1.88$; $P = 0.18$; Table 4.3) or total VFA concentration at dissection ($F_{2,22} = 2.26$; $P = 0.13$; Table 4.3). In addition, probiotic treatment did not affect acetate ($F_{2,24} = 0.94$; $P = 0.41$; Table 4.3), propionate ($F_{2,20} = 0.95$; $P = 0.40$; Table 4.3), butyrate ($F_{2,20} = 0.86$; $P = 0.44$; Table 4.3), or acetate to propionate ratio ($F_{2,23} = 0.44$; $P = 0.65$; Table 4.3) at dissection.

Stereo and light microscopy of reticulorumen papillae for each *Megasphaera elsdenii* treatment is displayed in Figure 4.1. Briefly, probiotic treatment affected reticulorumen papillae length ($F_{2,24} = 6.04$; $P = 0.01$; Table 4.3) as ME_{15} calves had greater papillae length than ME_0 and ME_{15+39} (Table 4.3). No differences in papillae length were observed between ME_0 and ME_{15+39} (Table 4.3). Probiotic treatment also affected papillae width ($F_{2,24} = 3.59$; $P = 0.04$; Table 4.3) as ME_{15} calves tended to have greater papillae width

than ME_0 and ME_{15+39} (Table 4.3). No differences in papillae width were observed between ME_0 and ME_{15+39} (Table 4.3). Reticulorumen papillae area was also affected by probiotic treatment ($F_{2,24} = 5.39$; $P = 0.01$; Table 4.3) as ME_{15} calves had greater papillae area than ME_0 calves and tended to have greater area than ME_{15+39} calves (Table 4.3). No differences in papillae area were observed between ME_0 and ME_{15+39} calves (Table 4.3). Lastly, probiotic treatment did not affect the thickness of the strata spinosum and basale ($F_{2,22} = 0.39$; $P = 0.68$; Table 4.3), granulosum ($F_{2,22} = 0.49$; $P = 0.62$; Table 4.3), or corneum ($F_{2,23} = 0.16$; $P = 0.86$; Table 4.3).

4.4 DISCUSSION

In this study, we evaluated the effects of an oral probiotic capsule containing *Megasphaera elsdenii* on reticulorumen pH, VFA concentration, and anatomical development of dairy-beef crossbred calves. The results from this study indicate that there might be benefits to utilizing a *Megasphaera elsdenii* capsule in dairy-beef crossbred calves during the preweaning period, with increased reticulorumen and papillae size, as well as changing VFA concentration. This study builds upon previous work showing the possible benefits of oral interventions with *Megasphaera elsdenii* on Holstein cows (Mazon et al., 2020) and calves (Muya et al., 2015). Yet, the effects of oral interventions with *Megasphaera elsdenii* on reticulorumen pH, VFA, and anatomical development of dairy-beef crossbred calves have not been previously evaluated.

We observed that probiotic treatment tended to affect reticulorumen pH throughout the whole experimental period, but we did not observe any individual differences between treatments. Previous research has shown that reticulorumen pH in calves seems to be lower

than in adult cattle, which agrees with the mean values obtained in the present study (Gentile et al., 2004, Yohe et al., 2015). When evaluating the long-term establishment of *Megasphaera elsdenii* in dairy calves, Yohe et al. (2018) did not report differences in reticulorumen pH between Holstein calves receiving a *Megasphaera elsdenii* drench versus control. However, when evaluating the effects of a *Megasphaera elsdenii* probiotic drench in mid-lactation dairy cows under a SARA challenge, Mazon et al. (2020) reported that cows receiving *Megasphaera elsdenii* had greater reticulorumen pH and less intense SARA when compared to control cows. In ruminants, high starch diets have been shown to favor the production of VFA and lactate in the reticulorumen (Nafikov and Beitz, 2007). This can be attributed to the observed increases in amylolytic bacteria populations, especially the acid-tolerant and lactate-producing *Streptococcus bovis* (Russell, 2002, Wang et al., 2015). Lactate is a stronger acid than VFA, thus lactate accumulation is associated rapid pH declines in the reticulorumen (Dijkstra et al., 2012). Lactate utilizing bacteria such as *Megasphaera elsdenii* play a vital role in reticulorumen pH maintenance as they are able to metabolize lactate into VFA that will be absorbed via the reticulorumen epithelium (Wang et al., 2015). In fact, *Megasphaera elsdenii* is highly successful competing for substrate, making it responsible for fermenting most of the lactate of dairy cattle under normal reticulorumen conditions (Counotte et al., 1981b). Still, the importance of *Megasphaera elsdenii* activity seems to be further accentuated under SARA conditions. In an in-vitro study, Chen et al. (2019) reported that *Megasphaera elsdenii* demonstrated populational growth and was able to utilize lactate at a faster rate than it was being produced under prolonged SARA conditions.

Low reticulorumen pH for prolonged periods of time has been associated with poor feed digestion (Cerrato-Sánchez et al., 2007) and epithelial health (Steele et al., 2011, Li et al., 2019). Yet, calves are especially susceptible to decreases in reticulorumen pH, during weaning as they transition from liquid to solid diets with elevated proportions of non-fibrous carbohydrates (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020). We hypothesized that calves receiving the *Megasphaera elsdenii* capsule would present greater reticulorumen pH compared to control. However, in the present study, probiotic administration tended to lower reticulorumen pH in supplemented calves. We hypothesize that these differences in reticulorumen pH might be associated with the greater solid feed DMI displayed by calves supplemented with the *Megasphaera elsdenii* capsule in the companion to this paper (Mazon et al., 2023). Furthermore, we believe that supplementation with *Megasphaera elsdenii* might have increased reticulorumen resilience to drastic drops in reticulorumen pH caused by the increased solid feed DMI, as similarly reported by Mazon et al. (2020) in dairy cows. Subacute ruminal acidosis pH thresholds are well defined in adult cattle (see review by Plaizier et al., 2008) yet, to our knowledge, no clear SARA thresholds have been defined for calves. Still, Meissner et al. (2017) reported that short exposure to reticulorumen pH and VFA concentrations similar to the ones observed in the present study were sufficient to damage tight junction proteins and disturb epithelial barrier function in sheep. Thus, future research should utilize indwelling reticulorumen pH measurements systems (Penner et al., 2009) to evaluate reticulorumen pH in calves and its relationship to nutritional interventions, such as providing *Megasphaera elsdenii* probiotics.

We observed that calves given two doses of the probiotic had greater reticulorumen propionate concentration throughout the experimental period compared to calves receiving either a placebo or single dose of the probiotic capsule. Furthermore, the calves receiving two capsules of the *Megasphaera elsdenii* probiotic tended to have greater total reticulorumen VFA concentration and greater reticulorumen concentrations of acetate over the whole experimental period compared to a placebo control. The results obtained for reticulorumen VFA concentrations are within the range reported by recent studies evaluating *Megasphaera elsdenii* in dairy calves (Muya et al., 2015, Yohe et al., 2018). In contrast to the present study, Yohe et al. (2018) did not observe any differences in reticulorumen VFA concentrations between calves receiving a *Megasphaera elsdenii* drench and control when evaluating long-term establishment of *Megasphaera elsdenii* populations in dairy calves. Muya et al. (2015) also reported no differences in total reticulorumen VFA concentrations in dairy calves between control calves and calves receiving an oral dose of *Megasphaera elsdenii* at 14 days of age. However, the same authors reported that dairy calves receiving the *Megasphaera elsdenii* probiotic had greater reticulorumen butyrate concentrations compared to control calves, which was not observed in the present study. Neither Muya et al. (2015) nor Yohe et al. (2018) reported differences in reticulorumen propionate concentrations between dairy calves receiving a *Megasphaera elsdenii* oral probiotic and control calves. Greater reticulorumen propionate concentrations have been observed in dairy heifers fed high-grain postweaning diets (Rosadiuk et al., 2021). As reported in the companion to this paper, no differences in solid feed DMI were observed between ME_{15} and ME_{15+39} calves (Mazon et al., 2023). Thus, no differences in VFA concentrations were expected between ME_{15} and ME_{15+39}.

The production and absorption of VFAs provides the chemical stimulus needed for the development of the reticulorumen epithelium (Sander et al., 1959, Tamate et al., 1962, Diao et al., 2019). Still, the ability to stimulate reticulorumen development in calves differs between VFAs and is highly associated with the concentrations of butyrate, followed by acetate and propionate, respectively (Bergman, 1990). As reviewed by Khan et al. (2011b), diets containing greater proportions of non-fibrous carbohydrates favor the production of butyrate and propionate, whereas as the inclusion of forages promotes acetate production. Still, fermentation of diets containing elevated proportions of non-fibrous carbohydrates might result in VFA accumulation and lactate production causing rapid pH declines in the reticulorumen (Laarman and Oba, 2011). However, *Megasphaera elsdenii* is known for utilizing reticulorumen lactate and producing propionate via the acrylate pathway (Mackie and Gilchrist, 1979). Thus, we hypothesize that providing a second capsule of *Megasphaera elsdenii* before weaning might have caused shifts in the reticulorumen microbiome, favoring the production of acetate and propionate (Hino et al., 1994, Weimer and Moen, 2013, Arik et al., 2019). Hence, future research should investigate the effects of the number and timing of *Megasphaera elsdenii* capsule administration on reticulorumen microbiome and its association with reticulorumen VFA.

In addition to VFA production and absorption, the physical presence of solid feed also plays a role in reticulorumen development has been associated with greater reticulorumen weight and capacity (Tamate et al., 1962, Khan et al., 2008, Khan et al., 2011a). In the present study, probiotic treatment affected empty reticulorumen weight as ME_{15} calves had greater empty reticulorumen weights compared to ME_0 calves and tended to have greater reticulorumen weights compared to ME_{15+39} calves. However, we did not

observe any effects of probiotic treatment on omasum or abomasum weights. We also observed that probiotic treatment tended to affect full reticulorumen weight. The forestomach weights observed in this study were within range for dairy calves dissected at similar ages (Khan et al., 2007b, 2008, 2011a). Solid feed intake is essential for reticulorumen growth and anatomical development in calves (Tamate et al., 1962, Baldwin et al., 2004, Khan et al., 2011b), thus it was expected that the greater solid feed DMI presented by calves receiving the *Megasphaera elsdenii* probiotic in the companion study (Mazon et al., 2023) would have greater reticulorumen sizes compared to placebo calves. Our findings concur with the results reported by Muya et al. (2015) where dairy calves receiving a *Megasphaera elsdenii* oral probiotic at 14 days of age had greater reticulorumen size compared to control calves when dissected at 42 days of age.

Solid feed DMI and the production and absorption of VFA have an effect on reticulorumen development. However, greater solid feed DMI and VFA uptake might also affect visceral organs. For instance, in ruminants, the majority of the glucose necessary for maintenance, growth, and production is produced in the liver from propionate via gluconeogenesis (Nafikov and Beitz, 2007). Furthermore, tissue developing and growth-promoting hormones, such as insulin-like growth factor-1 (IGF-1), are also produced in the liver (Bestetti et al., 1992, Cordano et al., 2000). Consequently, the liver has been classified as one of the main organs responsible for regulating metabolic pathways associated with nutrient utilization in cattle (Connor et al., 2010).

In the present study, probiotic treatment affected liver size, as calves receiving *Megasphaera elsdenii* had heavier livers compared to placebo calves. Previous researchers have reported positive associations between solid feed intake and liver metabolism

(Baldwin et al., 2004) and size in cattle (Hamada et al., 1976). Furthermore, in human medicine, the metabolic load hypothesis states that increases in metabolic load via the portal vein are responsible for triggering an increase on hepatocyte proliferation rates (Michalopoulos, 2007, Hohmann et al., 2014). The companion to this paper reported that calves receiving the *Megasphaera elsdenii* capsule displayed greater solid feed DMI and average daily gain than control calves (Mazon et al., 2023). And, as previously mentioned, ruminants rely on hepatic gluconeogenesis from propionate to meet their glucose needs (Nafikov and Beitz, 2007).Thus, we hypothesize that the observed differences in liver size observed in the present study, might be associated with the increased solid feed DMI reported in the companion paper (Mazon et al., 2023) and the probiotic effect on reticulorumen propionate concentrations observed in the present study. To our knowledge, the present study is the first to look at liver size in dairy-beef crossbred calves supplemented with *Megasphaera elsdenii*. Yet, in a study with beef steers, DeClerck et al. (2020b) reported no effects of oral *Megasphaera elsdenii* supplementation in liver abscess scores at dissection. Future studies should investigate the effects of *Megasphaera elsdenii* probiotic intervention on the hepatic function during the rearing period and investigate the possible long-term effects of the probiotic on the presence and severity of liver abscesses.

To our knowledge, the present study is the first to look at reticulorumen pH and VFA concentration at the point of dissection in dairy-beef crossbred calves supplemented with *Megasphaera elsdenii*. Yet, we did not observe any probiotic treatment effects on reticulorumen pH or VFA concentrations at dissection. We believe the elevated solid feed DMI presented by all treatments during the postweaning period reported in the companion to this paper (Mazon et al., 2023) might have contributed to the lack of differences between

treatments regarding reticulorumen pH and VFA concentration at dissection. Despite the lack of differences in reticulorumen pH and VFA concentration at dissection, probiotic treatment affected reticulorumen papillae size and area. We observed that ME_{15} calves had greater reticulorumen papillae length than ME_0 and ME_{15+39} calves. Furthermore, ME_{15} calves tended to have wider reticulorumen papillae compared to ME_0 and ME_{15+39} calves. Our results agree with Muya et al. (2015) who reported that dairy calves receiving an oral dose of *Megasphaera elsdenii* probiotic at 14 days of age had wider reticulorumen papillae than control calves. However, the same authors did not report any effects of *Megasphaera elsdenii* administration on reticulorumen papillae length. Papillae dimension is considered one of the main factors for evaluating reticulorumen development in calves (Lesmeister et al., 2004, Diao et al., 2019). In addition, papillae dimensions and surface area could be associated with increased VFA absorption capacity in calves (Yohe et al., 2019a, 2019b). Furthermore, recent studies reported that greater papillae dimensions have been associated with increased VFA absorption and increased ketogenic activity, possibly reducing SARA time in calves (van Niekerk et al., 2021, Nishihara et al., 2023). Subacute reticulorumen acidosis in calves has been associated with poor performance (Gelsinger et al., 2020) and poor reticulorumen epithelial health (Li et al., 2019). Like reticulorumen size, reticulorumen papillae development in calves can be associated with solid feed intake (Baldwin et al., 2004, Khan et al., 2007b). The companion to this paper reported that ME_{15} and ME_{15+39} calves had greater solid feed DMI than ME_0 calves (Mazon et al., 2023), thus we expected that both ME_{15} and ME_{15+39} would present similar reticulorumen papillae size and area. However, that was not observed in the current study. We hypothesize that providing calves with a second capsule of *Megasphaera elsdenii* might have affected shifts

in the reticulorumen microbiome, affecting reticulorumen VFA production, and therefore, reticulorumen papillae development. Future research should further investigate the effects of multiple *Megasphaera elsdenii* administrations on reticulorumen microbiome, VFA and papillae development in calves.

Although probiotic treatment affected the papillae length, width, and area in supplemented calves, it did not affect the papillae epithelial layer thickness between treatments. Li et al. (2019) reported that SARA affected pathways associated with cell division in calves, possibly affecting their reticulorumen epithelial health. Furthermore, Steele et al. (2011) reported reductions in the thickness of the stratum basale, spinosum, and granulosum, layers of the reticulorumen epithelium of mature dairy cows under a grain-induced SARA challenge. Therefore, we expected that calves receiving the *Megasphaera elsdenii* capsule would have more developed reticulorumen epithelium layers compared to control. Yet, differences in reticulorumen epithelium layers were not seen between treatments. Perhaps the age of the calves and time they were exclusively consuming solid feed in the present study might have not been enough to yield any visible differences in reticulorumen epithelium layers. For example, the results reported from Li et al. (2019) were from calves dissected at 17 weeks of age, whereas the calves enrolled in the present study were dissected approximately at 12 weeks of age. Still, it is important to highlight that reticulorumen epithelial health might be associated with the occurrence of liver abscesses. Damage to the reticulorumen epithelium has been associated with the incidence of liver abscesses, as they might allow microorganisms to enter the bloodstream (Nagaraja and Titgemeyer, 2007a, Elmhadi et al., 2022). Therefore, future research should

investigate the effects of early life *Megasphaera elsdenii* supplementation on long term reticulorumen epithelial health and liver abscess occurrence in cattle.

Overall, we observed that early-life supplementation of *Megasphaera elsdenii* probiotics increased the performance (Mazon et al., 2023), VFA concentration, and reticulorumen development of dairy-beef crossbred calves. Reticulorumen pH in calves seems to be lower than in adult cattle (Gentile et al., 2004, Suarez-Mena et al., 2016, Yohe et al., 2018). Yet, calf performance, and reticulorumen development seem to be negatively affected by SARA (Li et al., 2019, Gelsinger et al., 2020). These severe decreases in reticulorumen pH seem to happen mainly during weaning as calves transition from liquid to solid diets (Beharka et al., 1998, Suarez-Mena et al., 2016, Gelsinger et al., 2020). Still, the presence of milk in the reticulorumen may lead to SARA events in early life (Lorenz, 2009). As reviewed by Baldwin et al. (2004) and (Khan et al., 2016), inoculation and establishment of a healthy reticulorumen microbiome in conjunction with solid feed intake are fundamental for reticulorumen development in calves. In fact, previous researchers have suggested that probiotic supplementation in calves might aid the development of the reticulorumen microbiome, promoting reticulorumen development and helping calves transition from liquid to solid diets (Krehbiel et al., 2003, Diao et al., 2019). Our results demonstrate that early nutritional interventions with probiotics containing *Megasphaera elsdenii* seem to be beneficial for the growth and reticulorumen development of dairy-beef crossbred calves. Yet, it is still unclear whether providing calves with an additional *Megasphaera elsdenii* capsule around weaning might offer additional benefits regarding reticulorumen environment. Thus, future researchers should investigate how the timing of *Megasphaera elsdenii* administration affects reticulorumen microbiome. Lastly, there is an

opportunity to investigate the effects of early-life *Megasphaera elsdenii* supplementation on long-term performance, reticulorumen environment, and health in cattle.

4.5 CONCLUSION

The results from this study indicate that there might be benefits to utilizing a *Megasphaera elsdenii* capsule in dairy-beef crossbred calves. The *Megasphaera elsdenii* capsule tended to increase reticulorumen size and total VFA concentration. However, the number of capsule applications and its timing might be important factors to consider, as we observed differences in reticulorumen VFA concentration and papillae size and area between treatments receiving the *Megasphaera elsdenii* capsule. Future research should investigate the effects of the *Megasphaera elsdenii* capsule and time of application on the reticulorumen microbiome and VFA production in crossbred dairy calves.

Table 4.1 Least squares mean ±SEM of reticulorumen pH and VFA concentration for dairy-beef crossbred calves (n = 31) during the 76-day experimental period. Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study. Reticulorumen fluid samples were collected via esophageal tubing on days 14, 35, 49, 58, and 70 of the experimental period.

	Probiotic Treatment				*P*-value[1]			
Item	ME_0	ME_{15}	ME_{15+39}	SEM	Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Reticulorumen pH	6.12	5.92	5.83	0.29	0.10	0.32	0.11	0.46
Reticulorumen VFA								
Total VFA, mmol	86.77	102.23	106.43	21.74	0.07	0.17	0.08	0.60
Acetate, mmol	51.78	62.96	64.90	16.00	0.08	0.16	0.10	0.73
Propionate, mmol	25.55	28.23	30.68	3.53	0.04	0.34	0.03	0.34
Butyrate, mmol	10.51	12.81	11.85	2.56	0.36	0.47	0.77	0.77
Acetate to propionate ratio	2.33	2.47	2.29	0.32	0.54	0.87	0.87	0.85

[1] *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979)

Table 4.2 Least squares mean ±SEM of foregut and visceral organ weights for dairy-beef crossbred calves at dissection (n = 31; 86.2 ± 2.2 days old). Calves were assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study.

	Probiotic Treatment				*P*-value[1,2]			
Item	ME_0	ME_{15}	ME_{15+39}	SEM	Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Reticulorumen								
Full, kg	11.10	13.53	14.46	1.42	0.06	0.22	0.07	0.54
Full, % of BW	10.31	10.73	11.08	0.92	0.69	NS	NS	NS
Empty, kg	2.75	3.55	3.00	0.23	0.02	0.01	0.32	0.08
Empty, % of BW	2.38	2.65	2.28	0.17	< 0.01	0.01	0.25	< 0.01
Omasum								
Full, kg	0.70	0.82	0.87	0.13	0.36	0.68	0.50	0.69
Full, % of BW	0.62	0.58	0.66	0.08	0.64	NS	NS	NS
Empty, kg	0.48	0.47	0.50	0.07	0.85	NS	NS	NS
Empty, % of BW	0.46	0.39	0.39	0.06	0.22	0.35	0.35	0.92

Table 4.2 Continued

Item	Probiotic Treatment			SEM	*P*-value[1,2]			
	ME_0	ME_{15}	ME_{15+39}		Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Abomasum								
Full, kg	2.13	2.67	2.39	0.22	0.24	0.28	0.69	0.69
Full, % of BW	1.89	2.04	1.81	0.16	0.57	NS	NS	0.88
Empty	0.67	0.72	0.84	0.09	0.29	0.63	0.40	0.58
Empty, % of BW	0.65	0.61	0.63	0.07	0.85	NS	NS	NS
Visceral organs								
Liver, kg	2.12	2.60	2.71	0.19	0.02	0.07	0.02	0.64
Liver, % of BW	1.96	2.12	2.09	0.11	0.07	0.09	0.14	0.61
Kidneys	0.47	0.51	0.52	0.03	0.49	0.77	0.75	0.79
Kidneys, % of BW	0.43	0.41	0.40	0.01	0.12	0.30	0.14	0.59
Spleen, kg	0.26	0.28	0.29	0.02	0.71	NS	NS	NS
Spleen, % of BW	0.23	0.21	0.22	0.01	0.43	0.62	0.73	0.73

[1] *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979)

[2] NS represents a Bonferroni-adjusted *P*-value ≥ 1.0

Table 4.3 Least squares mean ±SEM of reticulorumen pH, reticulorumen VFA concentration, papillae dimensions and strata measurements for dairy-beef crossbred calves at dissection (n = 31; 86.2 ± 2.2 days old). Calves assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0), probiotic capsule on study day 15 (ME_{15}), or probiotic capsule on days 15 and 39 (ME_{15+39}) of the study.

	Probiotic Treatment				*P*-value[1,2]			
Item	ME_0	ME_{15}	ME_{15+39}	SEM	Treatment	ME_0 vs ME_{15}	ME_0 vs ME_{15+39}	ME_{15} vs ME_{15+39}
Reticulorumen pH	4.94	5.04	5.05	0.10	0.18	0.31	0.25	0.86
Reticulorumen VFA								
Total VFA, mmol	229.93	201.70	206.81	24.49	0.13	0.21	0.21	0.73
Acetate, mmol	131.75	116.90	117.99	19.53	0.41	0.74	0.74	0.93
Propionate, mmol	70.00	57.20	66.21	9.20	0.40	0.56	0.71	0.71
Butyrate, mmol	27.49	28.85	23.74	3.89	0.44	0.74	0.69	0.69
Acetate to propionate ratio	2.10	2.36	1.96	0.37	0.65	NS	NS	NS
Reticulorumen papillae measurements								
Length, mm	2.51	3.59	2.74	0.36	0.01	0.01	0.47	0.02
Width, mm	1.75	2.01	1.76	0.14	0.04	0.08	0.91	0.08
Area, mm^2	14.79	19.34	16.11	1.64	0.01	0.01	0.32	0.06
Papillae Strata measurements								
Stratum spinosum and basale, µm	32.53	34.80	33.00	2.30	0.68	NS	NS	NS
Stratum granulosum, µm	27.82	26.59	27.90	1.75	0.62	NS	NS	NS
Stratum corneum, µm	15.86	15.65	14.86	1.66	0.86	NS	NS	NS

[1] *P*-values were adjusted using the stepdown Bonferroni method of Holm (1979)

[2] NS represents a Bonferroni-adjusted *P*-value ≥ 1.0

Figure 4.1 Representative of the mean stereo and light micrographs of reticulorumen papillae from dairy-beef crossbred calves at dissection (n = 31; 86.2 ± 2.2 days old) for each treatment. Calves assigned to one of three probiotic treatments containing *Megasphaera elsdenii*: placebo (ME_0; A and D), probiotic capsule on study day 15 (ME_{15}; B and E), or probiotic capsule on days 15 and 39 (ME_{15+39}; C and F) of the study.

White bar, A, B, C = 1 mm.

Black bar, D, E, F = 50 µm**.**

Figure 4.1 A)

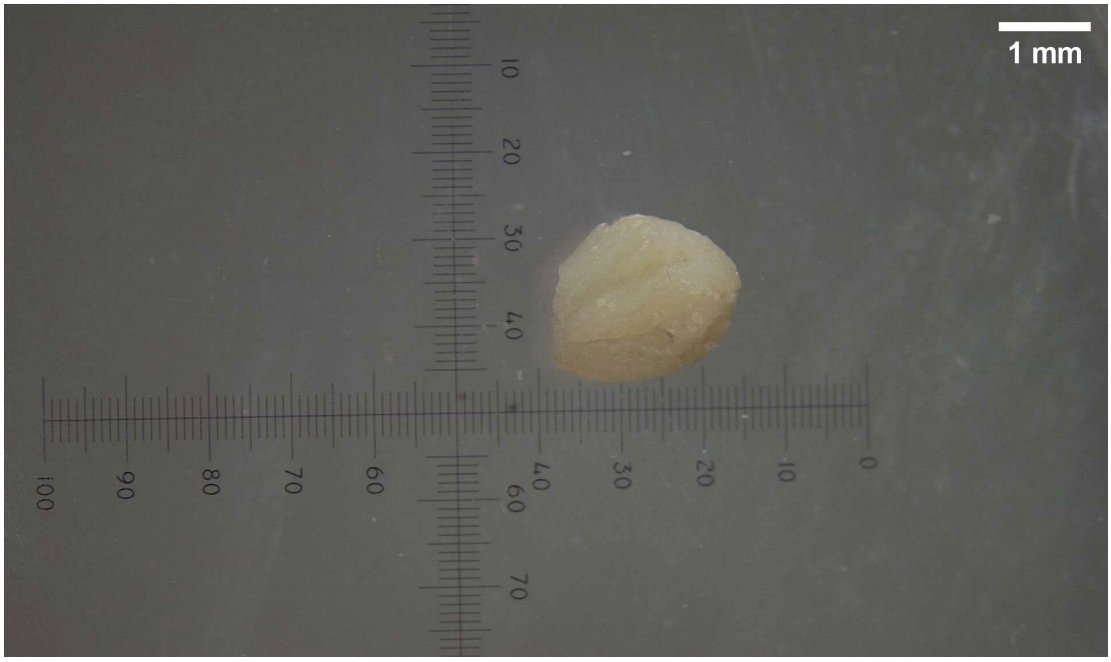


Figure 4.1 B)

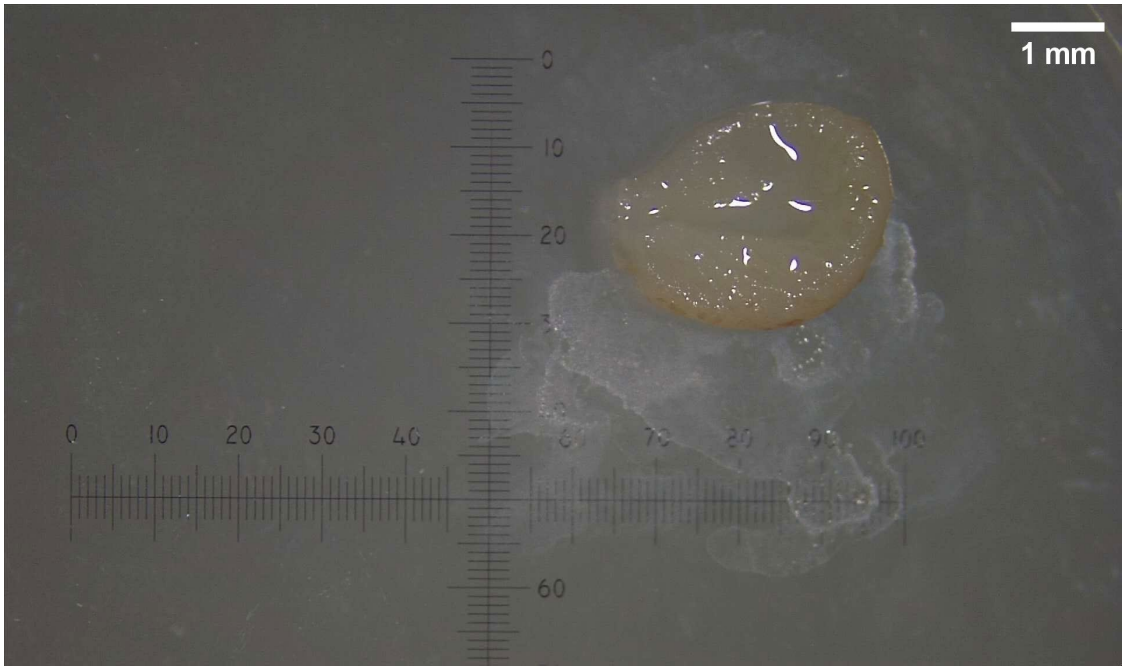


Figure 4.1 C)

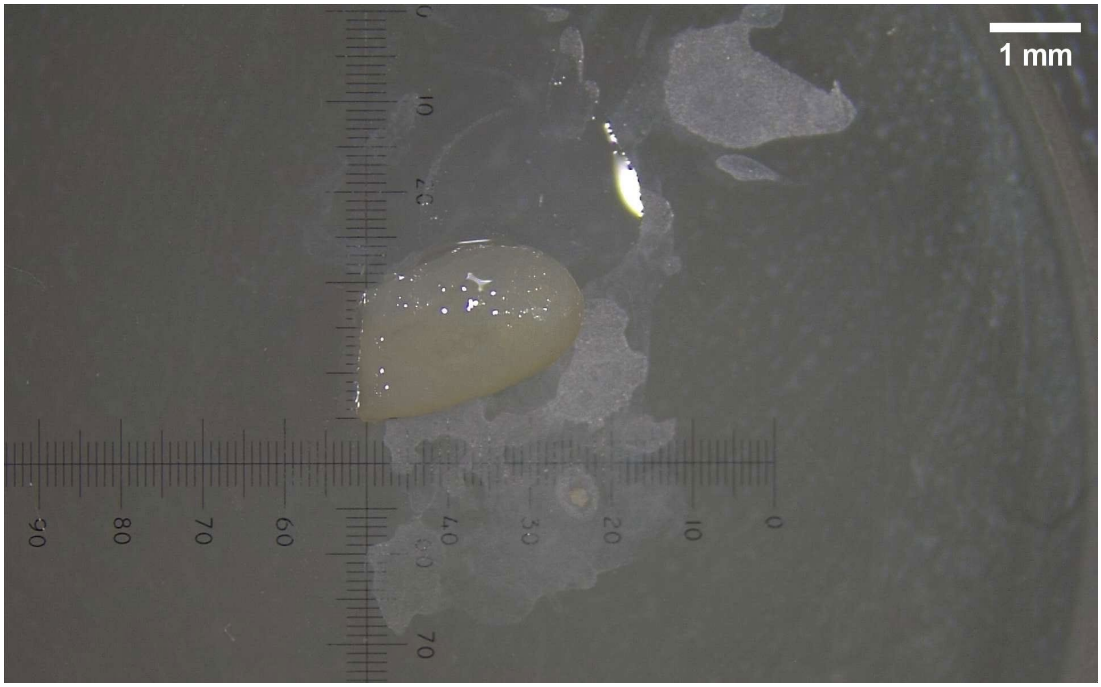
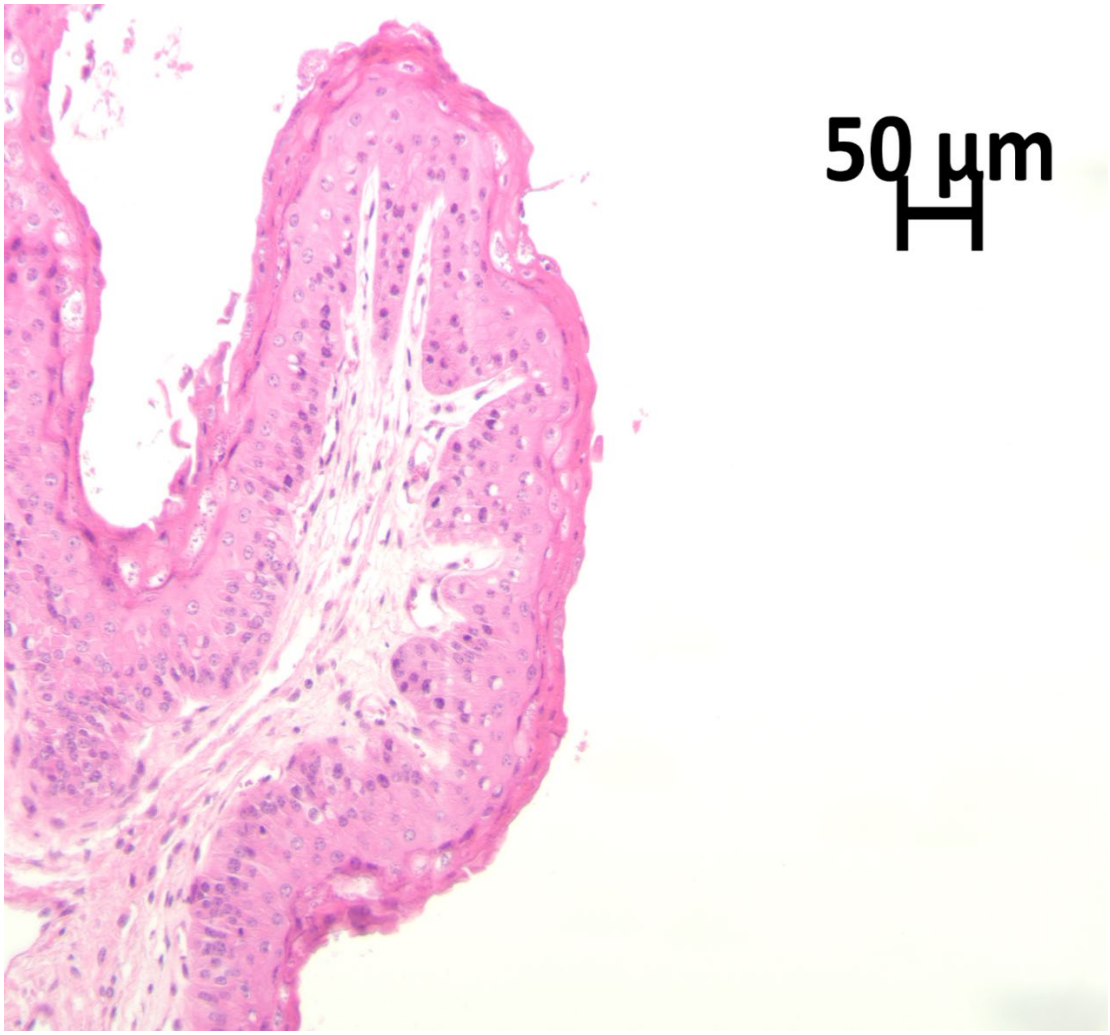


Figure 4.1 D)



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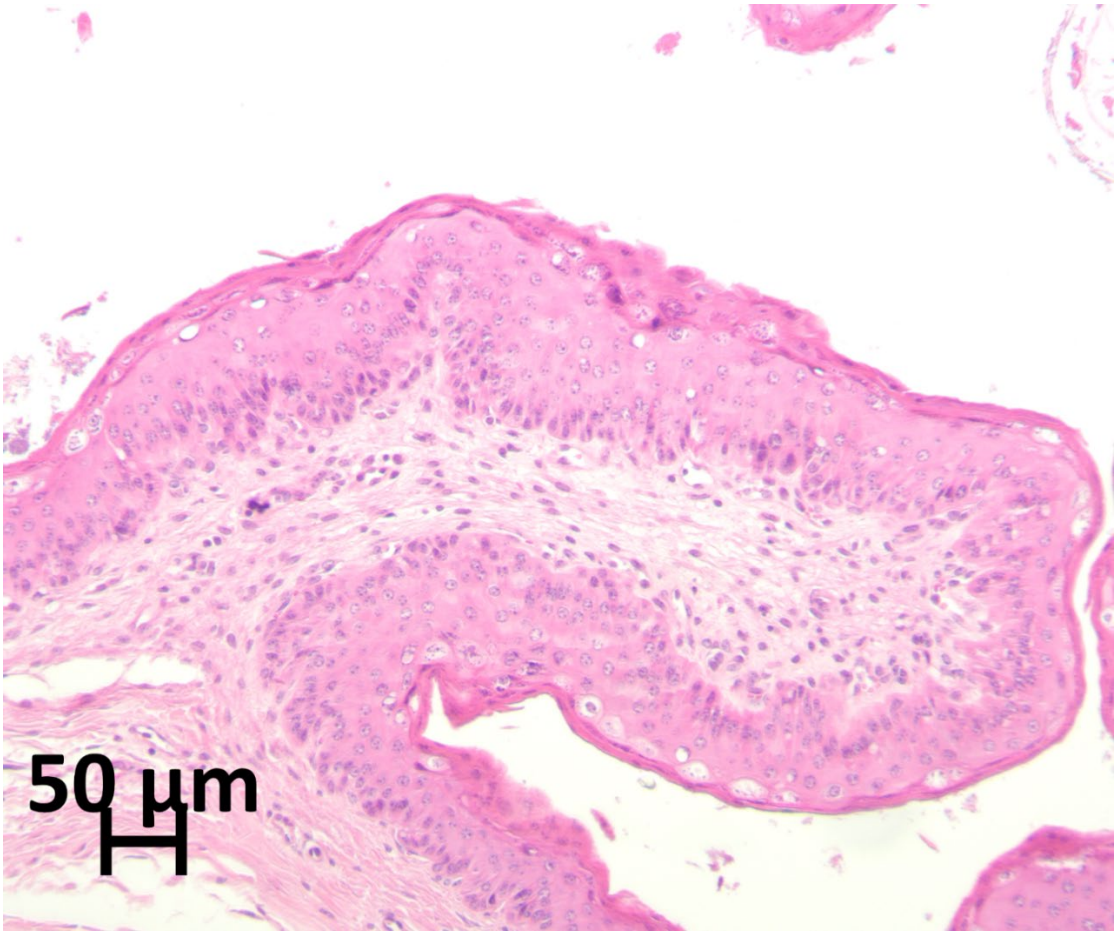
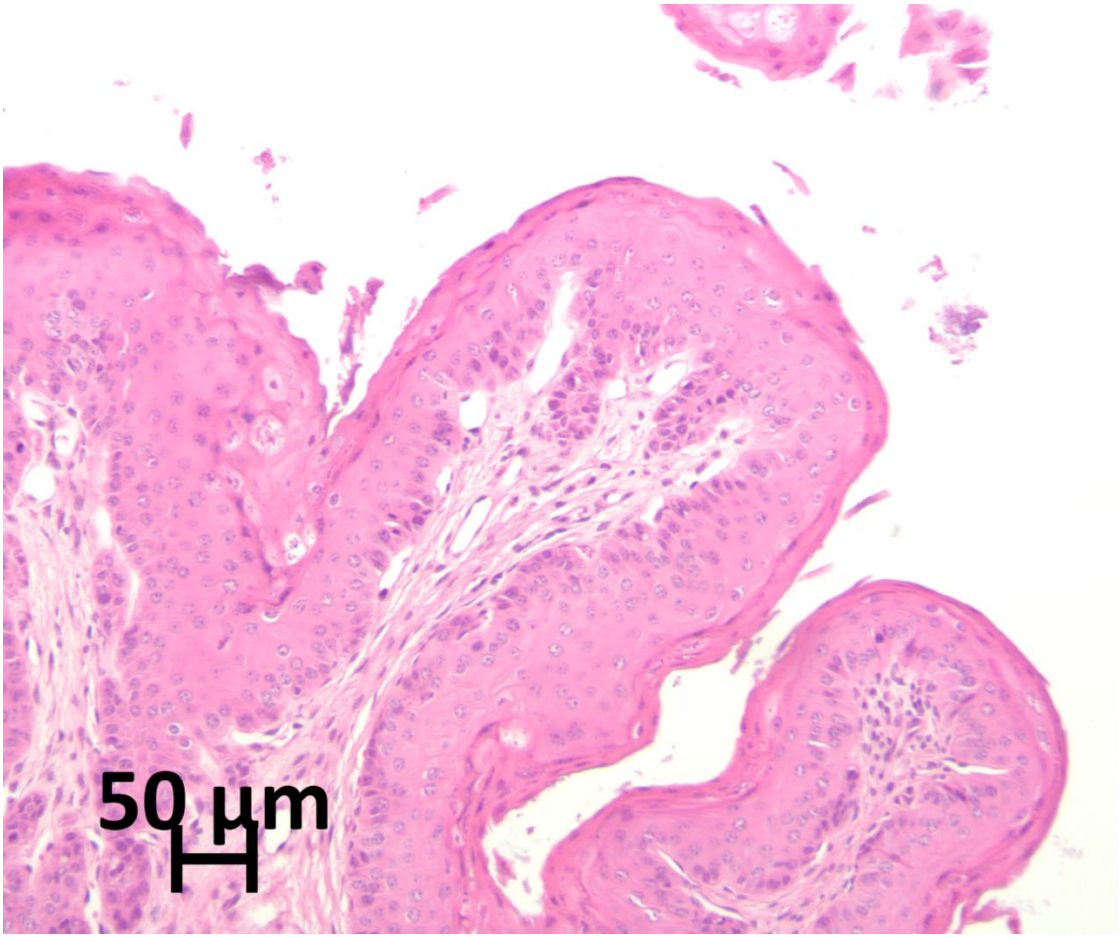


Figure 4.1 F)



CHAPTER 5. DISSERTATION GENERAL DISCUSSION

The contents of this dissertation served to further our understanding of the viability of utilizing *Megasphaera elsdenii* supplementation as a SARA prevention tool in cows and calves. We were also able to develop and evaluate effective and applicable strategic nutritional interventions utilizing *Megasphaera elsdenii* supplementation for cows and calves under SARA risk. In the review of literature, we discussed the importance of maintaining a stable reticulorumen pH. We also discussed that SARA is associated with abrupt changes to diets containing elevated proportions of non-fibrous carbohydrates, which is common during the transition period in cows and during the weaning period in calves. In addition, we discussed that SARA might be difficult to be diagnosed on farm and that it has negative effects on animal behavior, health, and performance. Then, we discussed that SARA can be prevented by increasing dietary fiber content or by including buffers, antimicrobials, or probiotics to the diet. The adoption of *Megasphaera elsdenii* supplementation has not only shown potential to be an effective strategy to prevent SARA as well provides us with an alternative solution for antimicrobial usage. However, there is limited research on the effects and proper utilization strategies of *Megasphaera elsdenii* supplementation. Hence, the objectives of this dissertation were to develop intervention strategies with *Megasphaera elsdenii* supplementation that are practical and effective to prevent SARA therefore improving cow and calf welfare and performance.

5.1 Summary of findings

Chapter 2 of this dissertation aimed to evaluate the effects of two different *Megasphaera elsdenii* supplementation strategies on reticulorumen pH, milk yield, and

feeding behavior of dairy cows under a SARA challenge. To accomplish the objectives proposed in Chapter 2 we conducted two crossover trials with 8 mid-lactation cows enrolled in each. The difference between each trial was the administration time of *Megasphaera elsdenii* supplementation in relation to a SARA challenge. In Trial 1, we administered an oral drench containing *Megasphaera elsdenii* 4 days before the SARA challenge, whereas in Trial 2 the oral drench was delivered the day before the SARA challenge. In trial 1 cows receiving *Megasphaera elsdenii* supplementation via oral drench experienced shorter and less intense SARA and displayed greater DMI and milk yield compared to control. We did not observe any effects of the *Megasphaera elsdenii* supplementation in trial 2. These results showed that the timing of the probiotic administration in relation to a SARA challenge may be essential for its efficacy, opening the door for further investigation in the topic.

We also developed two different strategic interventions with *Megasphaera elsdenii* supplementation for calves based on the results obtained in Chapter 2 and the limited literature on SARA prevention strategies for calves. The first strategy consisted in administering a probiotic capsule containing *Megasphaera elsdenii* to calves within the first weeks of life. The second probiotic intervention strategy consisted in administering a probiotic capsule containing *Megasphaera elsdenii* within the first weeks of life and then another capsule prior to the beginning of the weaning period. Then, we designed and conducted an experiment to evaluate these strategies.

Thirty-one dairy-beef crossbred calves in a randomized block trial divided into two blocks. Upon enrollment, calves were assigned to one of three treatments: an autoclaved probiotic capsule as placebo, a single administration of the probiotic capsule containing

Megasphaera elsdenii on day 15, or probiotic capsule administration on days 15 and 39 of the trial. Calves were individually housed and had free access to water and calf starter throughout the experimental period. Calves received 7 L/d of milk replacer that were split into two equal meals until day 41. Starting on day 42, calves received 3.5L/d of milk replacer divided into two meals until they were weaned on day 56. Starting on day 77, calves were euthanized and dissected for reticulorumen development assessment, anatomical measurements, and tissue sampling. During the trial wall-mounted cameras recorded calf feeding behavior and reticulorumen fluid samples were collected for pH and VFA analysis.

Chapter 3 of this dissertation aimed to investigate the effects of the different *Megasphaera elsdenii* supplementation strategies on feed intake, performance, and feeding behavior patterns of dairy-beef crossbred calves. We observed that both groups that received *Megasphaera elsdenii* supplementation displayed greater solid feed intake, average daily gain, and spent more time drinking water during the weaning and postweaning periods compared to control. Although we did not observe any differences in animal performance or behavior between the two different *Megasphaera elsdenii* supplementation treatments, we were unsure if the timing of probiotic intervention affected the reticulorumen environment of the caves.

Chapter 4 of this dissertation aimed to evaluate the effects of two different *Megasphaera elsdenii* supplementation strategies with an oral capsule on reticulorumen pH, VFA, and anatomical development of dairy beef-crossbred calves. We observed that control calves tended to have greater reticulorumen pH compared to calves receiving the *Megasphaera elsdenii* probiotic capsule. On the other hand, calves receiving the probiotic

capsule had greater reticulorumen propionate and displayed greater reticulorumen weight and papillae dimensions compared to control.

5.2 Implications and Future Directions

The results of these studies contribute to the current literature showing that SARA has negative impacts on cow and calf performance. More importantly, our results showed that we were able to develop and validate *Megasphaera elsdenii* supplementation strategies that was effective in preventing SARA as well as improved cow and calf performance without yielding any negative effects.

One of the many SARA prevention strategies adopted in the field consists of adding antimicrobials to the diet to decrease the population of pathogenic or lactate producing bacteria in the reticulorumen (Plaizier et al., 2018). In fact, the use of dietary antimicrobials is a common practice in cow (de Moura et al., 2021) and calf (Kertz et al., 2017) management systems. However, the vast adoption of antimicrobials in dairy management systems can lead to microorganisms developing antimicrobial resistance, which is a risk both for animal and human health (Rousham et al., 2018, Garcia et al., 2019).

The "One Health Approach" is a collaborative multidisciplinary effort to improve human, animal, and environmental health (Mackenzie and Jeggo, 2019). In fact, one of the key objectives of the "One Health Approach" is to halt the increases in antimicrobial resistant microorganisms, which can be achieved by increasing the efforts to develop alternatives to antimicrobial use such as vaccines, probiotics, and phytochemicals (Garcia et al., 2019). Therefore, it is important to highlight that the strategic *Megasphaera elsdenii*

supplementation to prevent SARA developed in this dissertation are an important step towards antimicrobial stewardship in dairy cattle production systems.

Furthermore, there is an opportunity to utilize data from precision livestock monitoring technologies to develop new *Megasphaera elsdenii* supplementation strategies for cows and calves. In the dairy industry, a few common examples of precision livestock technologies are wearable accelerometers, indwelling reticulorumen boluses, cameras, robotic milking systems, and automated milk/calf starter feeding systems (Stygar et al., 2021). These technologies are able to track and detect changes in animal performance and behavior helping producers make data-driven management decisions such as dietary changes, animal breeding, and disease treatment (Rojo-Gimeno et al., 2019). For example, Wagner et al. (2020) utilized data from an indwelling reticulorumen bolus and real-time locating system to develop a machine learning algorithm to detect dairy cows under SARA risk. In Chapter 2, we reported that providing cows with a probiotic containing *Megasphaera elsdenii* before inducing SARA resulted in decreased SARA time and intensity. Therefore, future research should investigate the efficacy of *Megasphaera elsdenii* supplementation based on SARA prediction algorithms from precision livestock monitoring technologies.

Solid feed intake data from automated calf starter feeding systems can be used to identify animals with abnormal solid feed intake and make management decisions, such as adjusting weaning age (de Passillé and Rushen, 2012, Benetton et al., 2019). Furthermore, Neave et al. (2019) reported that solid feed intake data from automated calf starter feeding systems such age to first solid feed ingestion and total solid feed intake preweaning can be used to identify calves that might struggle during weaning. We reported that calves

receiving *Megasphaera elsdenii* supplementation displayed greater solid feed intake and reticulorumen development. Therefore, there is an opportunity to use data from automated feeding systems to detect calves with low solid feed intake and perform a strategic *Megasphaera elsdenii* supplementation to promote solid feed intake and alleviate stress during the weaning period.

The development of an effective probiotic intervention requires knowledge of the disorder to be prevented as well as deep understanding of the microorganism to be utilized. The results of this dissertation showed that is possible to utilize *Megasphaera elsdenii* supplementation strategies to improve reticulorumen health and animal performance of cows and calves under SARA risk. However, there are still opportunities to investigate the economic viability of the strategies developed in this dissertation as well as their effects on the reticulorumen microbiome and long-term performance of cows and calves under SARA risk. Lastly, SARA is just one of the many disorders that cows and calves can experience throughout their productive cycle. Hence, there is an opportunity to utilize the concepts explored in this dissertation to develop and test new strategic nutritional intervention strategies to alleviate stress and improve overall health and welfare of cows and calves throughout their productive life.

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Zhang, T., Y. Mu, R. Zhang, Y. Xue, C. Guo, W. Qi, J. Zhang, and S. Mao. 2022. Responsive changes of rumen microbiome and metabolome in dairy cows with different susceptibility to subacute ruminal acidosis. Animal Nutrition 8:331-340.
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Zhou, M., Y. Chen, and L. Guan. 2015. Rumen bacteria. Rumen microbiology: from evolution to revolution:79-95.

VITA

1. EDUCATION

2019 **M.Sc. in Animal Sciences**
Focus: Dairy Cattle Nutrition, Behavior, and Health
University of Kentucky, Lexington, Kentucky, United States
Thesis: *Effects of yeast-derived microbial protein on transition dairy cow health and performance*
https://uknowledge.uky.edu/animalsci_etds/103/

2016 **B.Sc. Animal Sciences**
Federal University of Viçosa (UFV), Viçosa, MG, Brazil

2. PROFESSIONAL EXPERIENCE

Graduate Teaching Assistant (August 2019 – Present)
Department of Animal and Food Sciences, University of Kentucky, Lexington, KY

Graduate Research Assistant (August 2016 – August 2019)
Department of Animal and Food Sciences, University of Kentucky, Lexington, KY

Milk Quality Supervisor (December 2015 – July 2016)
Tirolez Cheeses, Quintinos, MG, Brazil

Data Quality Intern (August 2015 – December 2015)
Labor Rural, Viçosa, MG, Brazil

Dairy Consulting Intern (June 2012 – December 2015)
Dairy Activity Development Program, Viçosa, MG, Brazil

Milk Quality Intern (June 2012 – December 2015)
Southeast Quality Milk Initiative, Lexington, KY, United States

3. AWARDS & HONORS

3.1 Grants and Scholarships

(1) **Gustaf de Laval Fund**. 2022.
(2) **Merck Advancing Animal Welfare Together Research Showcase Graduate Student Travel Award**. 2022.
(3) **University of Kentucky Graduate Student Congress Travel Scholarship.** 2022.

(4) **National Milk Producers Federation Dairy Leadership Scholarship Program**. 2018.
(5) **University of Kentucky Student Government Grant**. 2018.
(6) **Brazilian Scientific Mobility Program Scholarship.** August 2014 to June 2015.

3.2 Awards

(1) **Second Place Northeast ARPAS Collegiate Research Contest**
Penn State Dairy Cattle Nutrition Workshop. 2023

(2) **First Place Graduate Student Award – Outstanding Overall Presentation**.
2nd Precision Livestock Farming Conference, 2023.

(3) **Fourth Place Graduate Team.** American Veterinary Medical Association Intercollegiate Animal Welfare Assessment Contest, 2022.

(4) **Third Place Graduate Team.** American Veterinary Medical Association Intercollegiate Animal Welfare Assessment Contest, 2021.

(5) **Third Place Graduate Team.** American Veterinary Medical Association Intercollegiate Animal Welfare Assessment Contest, 2019.

(6) **Second Place.** College of Agriculture, Food, and the Environment Three-Minute Thesis Competition, 2019.

(7) **First Place.** American Dairy Science Association Three-Minute Thesis Challenge, 2018.

(8) **Second Place.** American Dairy Science Association Southern Branch Dairy Production Oral Competition, 2018.

(9) **Third Place MS Student.** Tri-State Dairy Nutrition Conference, 2018.

4. PUBLICATIONS

4.1 Refereed Journal Articles

(1) Michalski, E., Woodrum Setser, M., **Mazon, G.**, Neave, H.W. and Costa, J.H., 2023. Personality of individually housed dairy-beef crossbred calves is related to performance and behavior. Frontiers in Animal Science, 3, p.161. https://doi.org/10.3389/fanim.2022.1097503

(2) Grinter, L.N., **Mazon, G.** and Costa, J.H.C., 2023. Voluntary heat stress abatement system for dairy cows: Does it mitigate the effects of heat stress on

physiology and behavior? Journal of Dairy Science, 106(1), pp.519-533. https://doi.org/10.3168/jds.2022-21802

(3) **Mazon, G.**, Montgomery, P.D., Hayes, M., Jackson, J., and Costa, J.H., 2021. Development and validation of an autonomous radio-frequency identification controlled soaking system for dairy cattle. Applied Engineering in Agriculture, 37(5), pp.831-837. https://www.doi.org/10.13031/aea.14344

(4) **Mazon, G.**, Campler, M.R., Holcomb, C., Bewley, J.M. and Costa, J.H.C., 2020. Effects of a Megasphaera elsdenii oral drench on reticulorumen pH dynamics in lactating dairy cows under subacute ruminal acidosis challenge. Animal Feed Science and Technology, 261, p.114404. https://doi.org/10.1016/j.anifeedsci.2020.114404

4.2. Peer-reviewed Extension Articles

(1) **Mazon, G.**, Amaral-Philiphs. D., Costa, J. H. C**.** 2018. "Getting the Most from Automatic Dairy Calf Feeders". Kentucky Dairy Notes, August 2018. (Print and Web).

(2) **Mazon, G**. and Amaral-Phillips D. M. 2015. Merits of Having First Lactation Dairy Cows in a Separate Management Group. Kentucky Dairy Notes.

4.3. Extension Publications

(1) Pereira, J. M. V., **Mazon G.**, and Joao H. C. Costa. 2022. Em busca de inovação: Tecnologias de Precisão e sua relação com bem estar animal e consumidores (*Seeking inovation – Precision dairy technologies and their relationship to animal welfare and consumer perception*) Revista Leite Integral. Brazil. July.

(2) **Mazon, G.** and Costa J. H. C. 2022. Assunto Ácido: Acidose Ruminal Subaguda em Bezerras (*A sore subject: subacute ruminal acidosis in calves*). Revista Leite Integral. Brazil. February.

(3) Costa, J. H. C., Reis M. E., **Mazon G.**, Cantor M. C., Neave H. H. 2020. Pensando Junto (*Thinking together: benefits of group and pair housing for dairy heifers*). Revista Leite Integral. Brazil. October.

(4) **Mazon, G.** and Costa J. H. C. 2018. Claudicacao em vacas leiteiras: um inimigo silencioso (Lameness in dairy cows: a silent enemy). Revista Leite Integral. Brazil. October.

(5) **Mazon, G.**, Amaral-Phillips D. M., and Costa J. H. C. 2018. Group Housing of dairy calves: Key management points. Progressive Dairyman. Canada. August.

(6) **Mazon, G.** and Costa J. H. C**.** 2018. Criacao Otimizada (Optimizing calf raising). Revista Leite Integral. Brazil. May.

(7) **Mazon, G.** and Costa J. H. C**.** 2018. O que Veremos no Futuro? (What is coming next regarding dairy technology?). Revista Leite Integral. Brazil. January.

(8) **Mazon, G.** and Bewley J. 2017. Go with the (cow) flow. Hoard's Dairyman. United States. May.

(9) **Mazon, G.** and Amaral-Phillips D. M. 2015. Why should I Have Two Groups of Dry Dairy Cows? Progressive Dairyman. October 19.