Dietary interventions to modulate gut function in ruminants

by

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B.S., Oklahoma State University, 2014
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Gastrointestinal health is exceedingly important in dairy cattle. Gastrointestinal inflammation in the gastrointestinal tract leads to a breakdown of the intestinal barrier, which allows pathogens and other infectious agents to enter the animal's body. Dairy calves undergo many physiological and environmental stressors during their first few months of life, which can cause the mucosal lining of the intestines to thin, thereby exposing the calf to pathogenic bacteria such as Clostridia species. Previous research has explored diet supplementation, including the use of direct fed microbials, to improve gastrointestinal health in dairy cattle. This dissertation focuses on addressing gastrointestinal health issues in two populations of dairy cattle: mid-lactation cows and calves. In the first study, mid-lactation dairy cows were supplemented with calcium gluconate with the goal of improving milk fat production as well as gastrointestinal health. Unfortunately, during the study, cows were exposed to several unplanned challenges including heat stress, mycotoxins (trichothecenes and zearalenone), and pathogenic bacteria (*Clostridium* species). Cows experienced sporadic intake and digestive upset, which likely contributed to reduced milk fat content (3.7% ± 0.20 at study enrollment declined to 3.4% ± 0.20 at the nadir). Cows with 3+ parities had greater ($P < 0.05$) dry matter intake (DMI) compared to 2nd lactation cows. Parity groups did not differ in milk fat content ($P > 0.05$), but 2nd lactations cows tended ($P = 0.098$) to have greater milk protein. Calcium gluconate supplementation had minimal effects on production. Supplementation tended ($P = 0.056$) to increase milk fat concentration (3.75 ± 0.050 vs. 3.66% ± 0.051), but also tended to reduce percent milk protein ($P = 0.08$) and lactose ($P = 0.07$) concentrations. Furthermore, there was a shift in milk fat composition. Cows supplemented with calcium gluconate had increased ($P < 0.05$) production of milk de novo synthesized and mixed-source fatty acids. In addition, plasma NEFA concentrations were elevated in supplemented cows ($P < 0.05$), but no other differences in blood

metabolites were observed ($P > 0.05$). In the second study, Holstein-Angus crossbred calves were randomly assigned to 1 of 2 treatments at birth and then were sacrificed at 30, 60, or 90 days of life. Treatments consisted of 1) a negative control milk replacer, or 2) a milk replacer supplemented with *Lactobacillus* and *Bacillus* species. No differences were found between control calves and probiotic supplemented calves for performance measurements ($P \geq 0.10$). There was a treatment × time interaction for starter DMI and total DMI ($P < 0.05$) with probiotic-supplemented calves having greater intakes for the first 30 d of the trial than control calves. Health scores suggested no differences between treatments ($P > 0.05$), with the exception of a tendency for ear score ($P = 0.08$) to be greater (indicative of more negative observations) in probiotic-supplemented calves compared to controls. No treatment effects ($P > 0.05$) were observed for plasma health biomarkers, intestinal tissue *E. coli* or other pathogen abundance, fecal *E. coli* or pathogen counts, or ileal histology. In this study, the combined probiotic delivered with milk replacer had little discernable benefit for calf health and growth, although this cohort of calves was not severely challenged. Across both studies, the interventions evaluated had few impacts on measures of gastrointestinal health, but documented changes in gastrointestinal physiology and barrier function in growing calves and in mature cows undergoing digestive problems provide valuable insights.

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concentrations were elevated in supplemented cows ($P < 0.05$), but no other differences in blood metabolites were observed ($P > 0.05$). In the second study, Holstein-Angus crossbred calves were randomly assigned to 1 of 2 treatments at birth and then were sacrificed at 30, 60, or 90 days of life. Treatments consisted of 1) a negative control milk replacer, or 2) a milk replacer supplemented with *Lactobacillus* and *Bacillus* species. No differences were found between control calves and probiotic supplemented calves for performance measurements ($P \geq 0.10$). There was a treatment × time interaction for starter DMI and total DMI ($P < 0.05$) with probiotic-supplemented calves having greater intakes for the first 30 d of the trial than control calves. Health scores suggested no differences between treatments ($P > 0.05$), with the exception of a tendency for ear score ($P = 0.08$) to be greater (indicative of more negative observations) in probiotic-supplemented calves compared to controls. No treatment effects ($P > 0.05$) were observed for plasma health biomarkers, intestinal tissue *E. coli* or other pathogen abundance, fecal *E. coli* or pathogen counts, or ileal histology. In this study, the combined probiotic delivered with milk replacer had little discernable benefit for calf health and growth, although this cohort of calves was not severely challenged. Across both studies, the interventions evaluated had few impacts on measures of gastrointestinal health, but documented changes in gastrointestinal physiology and barrier function in growing calves and in mature cows undergoing digestive problems provide valuable insights.

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Dedication

I would like to dedicate this dissertation to my parents, Tom and Paula, who have always been supportive and pushed me to challenge myself.

Preface

The small intestine wall contains several components that protect the gut from the microbes that inhabit the digestive tract. There are two types of barriers protecting the intestinal wall: physical and chemical (Okumura and Takeda, 2018). Physical barriers consist of a viscous mucus secreted by goblet cells that covers the intestinal mucosa. Within this mucus layer are antimicrobial peptides and regenerating islet-derived 3 (Reg3) molecules produced by Paneth cells in the epithelium. These chemical barrier molecules work to inhibit intestinal microbes from breaking down and penetrating the intestinal wall. Furthermore, tight junction barriers are proteins in between the epithelial cells that prevent invasive bacteria from entering the blood stream (Okumura and Takeda, 2018).

Animals experience several stressors which can diminish intestinal barrier function. Transportation of animals is a stressor that has been shown to impair the intestinal barrier (Meddings and Swain, 2000). Moreover, simply restraining and handling animals has been reported to reduce barrier function of the intestine (Wilson and Baldwin, 1999). Heat stressed dairy cattle have been reported to experience diminished intestinal barrier function (Baumgard et al., 2015); therefore, heat is a stressor that producers are always working to manage in livestock production. Heat stress is caused by a form of hypoxia due to blood being redirected to peripheral parts of the body to in attempt to cool itself (Pearce et al., 2013). Furthermore, mycotoxins in feed reportedly alter gut function of cattle and other animals. Diets containing deoxynivalenol (DON) were observed to have greater paracellular passage in swine (Pinton et al., 2010) and poultry (Awad et al., 2019), which indicates that the tight junction barriers were broken down, thereby allowing particles to enter the blood stream. In lactating dairy cows, exposure to mycotoxins, opportunistic pathogenic bacteria, and highly fermentable carbohydrates in the hindgut can lead to a breakdown of the intestinal tract. Moreover, calves

that have been in a negative energy balance have an increased gut permeability (Devant and Marti, 2020). Calves, specifically, experience many environmental and physiological stressors within their first few months of life (Hulbert and Moisá, 2016). From birth until a couple months of age, dairy calves in commercial practices are transported, dehorned, and commingled with other calves. These are all environmental stressors that can tax a calf's immune system (Hulbert and Moisá, 2016). Furthermore, calves, when born, function as non-ruminants and the rumen, reticulum, and omasum develop over the first few months of life (Heinrichs and Lesmeister, 2005). This shift in the digestive tract is associated with the calf transitioning from liquid to solid feed. As the calf consumes solid feed it enters the intestinal tract to be broken down and absorbed until the rumen has developed enough to serve as a fermentation vessel for nutrients (Baldwin et al., 2004). This combination of physiological and environmental stressors challenges the intestinal barrier.

Opportunistic pathogenic bacteria, such as *Clostridium perfringens*, *Salmonella*, and *Escherichia coli* (generic and pathogenic), are microorganisms that naturally inhabit the gastrointestinal tract (Bien et al., 2013; Christofi et al., 2019). They are present at relatively moderate levels in the gastrointestinal tract and generally do not harm the animal if the gut barrier is healthy. However, when the animal experiences a form of stress and the gut barrier begins to breakdown (Söderholm and Perdue, 2001), these opportunistic pathogens can become dangerous. Specifically, *Clostridium perfringens* can cause hemorrhagic bowel syndrome (HBS; Owaki et al., 2015). This syndrome causes the animal to hemorrhage internally in the jejunum of the small intestine. This occurs because *Clostridium perfringens* irritates the intestinal cells causing inflammation, then bleeding, and finally death (Owaki et al., 2015).

Research has shown that supplementing ruminant diets can improve gut function. More specifically, butyrate has reportedly been used as energy for epithelial cells which improves gut health (Guilloteau,et al., 2010; Penner et al., 2011) and supplementing animals with calcium gluconate has been reported to increase fecal butyrate (Tsukahara et al., 2002). In addition, butyrate has been associated with an increase in milk fat in cows (Izumi et al., 2019). The intestinal tract of calves can also be modified in a similar way. More specifically, direct fed microbials, such as *Lactobacillus* and *Bacillus*, have been observed to improve intestinal health of various animals by competing for resources with pathogenic bacteria (La Ragione and Woodward, 2003; Chen et al., 2007). This competitive exclusion reduces the pathogenic population (Nurmi et al., 1992; Steer et al., 2000) which reduces the severity of clinical disease (Callaway et al., 2008). The following chapters discuss potential strategies for modulating the gut to improve intestinal function to protect the animal from opportunistic pathogenic bacteria.

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Nutritional management effect on calf growth and health during the pre-weaning and weaning phases: A review.

S.T. Quanz, S. E. Gragg, and B. J. Bradford

INTRODUCTION

Calf rearing is a complex and integral part of the dairy industry. Calves are exposed to a series of challenges within their first few months of life (Hulbert and Moisá, 2016). Within the first few hours of life, calves are separated from their dams (Daros et al., 2014). Once separated, calves are usually provided with colostrum in the first 12 h of life, then supplemented either non-saleable milk or milk replacer 2-3 times daily. Often, in larger operations, calves are transported to a calf grower within the first week after being born. During this time calves are exposed to several different varieties of pathogenic bacteria within the first few days of life (Cho and Yoon, 2014). Many of these pathogens are enteric bacteria that cause calves to have neonatal diarrhea (scour), and in severe cases, lead to death (Cho and Yoon, 2014). After arrival to the grower, calves are typically comingled with other calves at approximately 3 weeks of age. With comingling disease spread is more prevalent (Frizzo et al., 2018) due to being in close contact with calves that may be carrying infectious pathogens (Barrington et al., 2002). During this stage of a calf's life, calf starter is introduced and gradually increases as a nutrient source until calves are weaned onto just solid feed. In beef cattle production, calves are typically weaned between 6 – 8 months of age when their rumens are completely functional, but in dairy cattle production, calves are typically weaned at 3 months or less in an attempt to manage labor and feed costs. This creates a challenge for calves to develop a functional rumen within the first few months of life. As a result of these multiple stressors, dairy calves often experience suboptimal growth and morbidity challenges. This review will focus on nutritional management strategies to mitigate some of these challenges and improve calf performance and health.

GESTATION PHASE

FETAL PROGRAMMING

In the past few years there has been an increase in publications focusing on fetal programming or the effect of maternal nutrition and health on the neonate (Vonnahme, 2007; Zhang et al., 2019; Huber et al., 2020; Osorio, 2020; Abuelo, 2020). Given this new knowledge, we will briefly discuss how maternal nutrition and health affects the neonate during pregnancy.

The ability of the dam to allocate resources to the fetus during pregnancy is dependent on the nutrition of the dam herself (Gaccioli et al., 2013). In late gestation fetal nutrient requirements take priority over the cow's (Bauman and Currie, 1980). Restricted maternal nutrition results in calves with reduced post-natal growth (Abuelo, 2020). In addition, these calves have diminished immune function, which leads to increases in morbidity and mortality (Cooke, 2019). On the other end of the spectrum, there are potentially negative effects of overfeeding cattle during late gestation. When the dam has increased energy intake during late gestation it has been shown to affect birth weight and immune function (Osorio et al., 2013). In rats, offspring of obese mothers had transcriptional patterns reflecting upregulated lipogenesis and adipogenesis (Borengasser et al., 2011). Even though this field needs more research, there is potential that over conditioned cows at parturition can lead to increased potential for metabolic health issues in offspring (Opsomer et al., 2016).

There have been indications that a cow's health and stress during gestation influences its offspring. Heat stress is constantly a concern in livestock management with multiple published works focusing on heats effect on production (Kadzere et al., 2002; West, 2003; Polsky and von keyserlingk, 2017), but there is also concern for carry-over effects of metabolic stress onto the offspring (Abuelo et al., 2020). Before the calf is even born it is being impacted by the health of the dam. The last 2 months of gestation account for 60% of the fetus's growth (Bauman and

Currie, 1980), and heat stressed cows have shorter gestation lengths (Adin et al., 2009; Tao et al., 2011; Tao et a., 2012). In addition, heat stress during gestation may cause increased fetal body temperature which can slow growth (Bell et al., 1989). This impact can even have a lasting effect after the calf is born. Almoosavi et al. (2020) found that cows that were heat stressed in the last 45 d of gestation produced calves with smaller birth weights and hip heights compared to cows that were thermoneutral during gestation. In addition, offspring from heat-stressed cows tended ($P = 0.09$) to have greater incidence of abnormal or loose feces (Almoosavi et al., 2020). It has been determined that the nutrition, health, and exposure to stress in gestating cows can have lasting effects of calf performance and health. Moving forward, we need to determine the best methods to handle these management challenges that in turn benefit the calf.

PRE-WEANING PHASE

COLOSTRUM QUALITY

Colostrogenesis begins within the mammary gland a few weeks before calving (Brandon et al., 1971). Prolactin and other lactogenic hormones begin this process, which then ceases immediately following parturition (Foley and Otterby, 1978). Colostrum is mainly composed of nutrients, growth factors, leukocytes, immunoglobulins (Ig), hormones, cytokines, and antimicrobial factors (Godden, 2008). Colostrum contains approximately 24% solids, which is almost twice as much as regular milk at 12.9% (Foley and Otterby, 1978; Hammon et al., 2013). This increase in solids is mainly due to a higher concentration of protein (14.0%) and fat (6.7%) in colostrum. This increase in nutrients is imperative for the establishment of the calf's immune system and thermogenesis (Foley and Otterby, 1978). Maternal leukocytes in colostrum mainly consist of T and B lymphocytes, macrophages, and neutrophils (Larson et al., 1980; Le Jan, 1996). Calves fed colostrum containing maternal leukocytes were observed to have cell-mediated

immune response by 1 wk of age compared to 3 wk for calves fed a leukocyte free colostrum (Reber et al., 2005).

Many maternal factors affect the composition and quantity of colostrum produced, such as length of the dry period, late-gestation nutrition, parity, and vaccination status (Godden, 2008; Godden et al., 2019). Length of the dry period has significant impact on colostrum quality. Cows that received no dry period had lower colostrum protein, IgG (Rastani et al., 2004; Shoshani et al., 2014), and IgM (Mayasari et al., 2015) concentrations compared to cows with a short (28 - 30 d) or traditional (56 - 60 d) dry period (Rastani et al., 2004; Shoshani et al., 2014; Mayasari et al., 2015). Immunoglobulin concentrations were similar between short and conventional dry-off periods (Shoshani et al., 2014; Mayasari et al., 2015). A decrease in IgG concentration would result in calves being required to consume more colostrum in order to meet their immunoglobulin requirements. This is compounded by the fact that cows with no dry period produced less colostrum than cows with short or conventional dry periods (5.1, 5.3, and 7.7 ± 0.6 kg, respectively; Mayasari et al., 2015).

Gavin et al. (2018) reported that primiparous cows produced more colostrum than multiparous cows in a cohort study on a commercial dairy in Texas. This is surprising as the general perception among producers is that multiparous cows produce more and higher quality colostrum than primiparous cows. This is more aligned with earlier research which reported that 1st and 2nd lactation cows produce colostrum with lower specific gravity than 3rd or 4th lactation cows (1.0483 ± 0.0007 and 1.0485 ± 0.0008 vs. 1.0516 ± 0.0009 and 1.0509 ± 0.0009, respectively; Morin et al., 2000). More recently, Shivley et al. (2018) observed IgG concentrations among cows of different parities and reported that cows with 3 or more (3+) lactations had greater IgG concentrations (84.7 ± 2.26 g/L) compared to 1st (72.3 ± 2.33 g/L) and

2nd (73.0 ± 2.40 g/L) lactation cows. In addition, parity 3+ cows had a higher percentage (80.4%) of excellent colostrum (>50 g/L IgG) than 1st and 2nd lactation cows (75.4 ± and 70.8%, respectively). Regardless, colostrum quality measurements should be taken on younger cows to ensure adequate high-quality colostrum is retained for newborn calves.

It is well established that prepartum nutrition is essential for cow health and performance during lactation. There is less published literature focusing on prepartum nutrition effects on colostrum quality. Mann et al. (2015) studied diet energy levels of cows during the dry period and its effect on colostrum. Cows fed a diet that met nutrient requirements at ad libitum intake (Control) had an average IgG concentration of 96.1 (83.3 – 108.9) g/L which was greater ($P <$ 0.05) than that from cows fed a diet that met 150% of nutrient requirements at ad libitum intake (72.4, 60.3 – 84.5, g/L; High). Control cows had lower concentrations of insulin ($P < 0.05$) compared to the High treatment. In addition, High cows tended ($P = 0.08$) to have greater colostrum fat yield (384, 295 – 473, g) than control cows (289, 196 – 380, g). Source of energy in a diet has potential to play a role in colostrum quality as well. Fatahnia et al. (2012) replaced corn with wheat in a diet for cows approximately 27 d before calving. Cows fed the diet with wheat as the energy source had greater protein concentration (13.70 vs. 12.99 ± 0.17%) in colostrum compared to cows fed the corn-based diet. Fatahnia et al. (2012) also observed that cows fed a diet containing wheat produced colostrum with greater total solids (25.20 vs. 23.96 ± 0.39%) and total IgG (106.33 vs. 79.72 ± 6.14 g/L) concentrations than the control group.

Beyond energy, supplementing a variety of other nutrients in the prepartum period has impacted colostrum quality. Supplementing rolled sunflower seed at 8% of diet DM compared to an equal amount of rolled canola or a basal diet not supplemented with oilseeds increased the Brix score of colostrum, indicative of greater solids content (Salehi et al., 2016). Nicotinic acid

supplementation also increased colostrum IgG and total protein concentrations compared to a negative control (Aragona et al., 2016; Aragona et al., 2020). Supplementing diets with β-carotene did not alter colostrum IgG concentration, but it did increase total solids concentration (Aragona et al., 2021).

Vaccinations during the prepartum period have been commonly used to boost the cow's immune system and in turn improve antibody transfer to the calf. Vaccinations for *E. coli*, rotavirus, and coronavirus have been utilized to protect the cow against mastitis and limit potential scours in offspring (Bürki et al., 1986; Hogan et al. 1992). Manichetti et al. (2021) evaluated responses to vaccinations at the start of the close-up period (21 d before parturition) compared to 7 d earlier (28 d before calving). Cows vaccinated approximately 28 d before calving had greater colostrum IgG concentrations (160.4 ± 7.0 g/L) than cows vaccinated at 21 d prepartum (134.4 ± 7.0 g/L). Conversely, cows vaccinated closer to calving had greater serum IgG concentrations (32.2 ± 1.2 g/L) than cows in the 28 d treatment (29.1 ± 1.2 g/L; Manichetti et al., 2021). This shift in IgG from serum to colostrum demonstrates the effect of vaccination timing on maternal immune function and ability to transfer immunoglobulins into colostrum for the calf.

When colostrum is unavailable, colostrum-derived replacers or plasma-derived replacers have been used to provide newborn calves with the passive immunity required to survive. Maternal colostrum has been reported to provide calves with greater total protein and immunoglobulins in blood than either colostrum-derived or plasma-derived replacers (Swan et al., 2007; Priestley et al., 2013; Lopez et al., 2020). This is most likely from the increased immunoglobulin absorption of maternal colostrum was compared to colostrum-derived (Smith and Foster, 2007; Lago et al., 2017; Lopez et al., 2020) and plasma-derived replacers (Priestley

et al., 2013). This increase in passive transfer translates to decreased morbidity and even tends to decrease mortality rates (Priestley et al., 2013). Furthermore, there is evidence that maternal colostrum allows for greater ADG and weaning weights (Lago et al., 2017), but those outcomes are not consistent (Lopez et al., 2020). More research is needed to solidify the concept that maternal colostrum increases performance in pre-weaned calves.

COLOSTRUM MANAGMENT

Colostrum management is arguably one of the most important aspects of newborn calf care (Godden et al., 2019). Calves are born without a working immune system and colostrum provides the necessary immunoglobins for passive immunity to be established (Godden, 2008; Godden et al, 2019). Time of colostrum administration, storage and method of administration are all factors that influence how well passive immunity is established.

When a calf ingests colostrum, it begins the process of taking up immune cells and molecules necessary to establish some degree of immunity to pathogens in its environment. The small intestine is the main section of the digestive tract responsible for immunological cell uptake (Corley et al., 1977); however, calves only have the ability to effectively take up intact immunoglobulins, leukocytes, and cytokines for approximately 24 h after birth (Stott et al., 1979; Barrington and Parish, 2001). This uptake occurs through a process called pinocytosis (Broughton and Lecce, 1970) which allows the calf to translocate macromolecules across the intestinal epithelium (Stott et al., 1979). Research has established that there is a steady decrease in a calf's ability to absorb macromolecules during the first 24 h of life (Stott et al., 1979; Fischer et al., 2018). Fischer et al. (2018) gave colostrum to calves at either 0, 6, or 12 h of life. Calves that received colostrum within the first hour of life had greater serum IgG concentrations for at

least the first 48 h of life. There was no difference in IgG concentrations between calves given colostrum at 6 h or 12 h (Fischer et al., 2018). As a result, it is generally accepted within industry to give calves colostrum as soon as possible.

Current conventional standards state that a newborn calf should consume approximately 10% of its birth weight in colostrum in order to receive the necessary antibodies needed to provide effective immune protection as the calf's own adaptive immune system is developing. Therefore, a 36-kg calf should consume 3.6 kg (~ 4 L) of colostrum within the first 24 h of life. In order to ensure calves receive the appropriate volume of colostrum, esophageal feeding tubes are often used. However, when feeding tubes are used, the esophageal groove is not activated, which causes the colostrum to be deposited in the reticulo-rumen. This causes a delay (~3 h) before the colostrum reaches the intestines for absorption (Lateur-Rowet and Breukink, 1983). However, even with this delay, passive transfer is still achieved when sufficient levels of colostrum are given (Desjardins-Morrissette et al., 2018). Sometimes esophageal feeding is required because a calf will not consume all 4 L of colostrum on its own within the first 12 h of life. There is one known study that specifically evaluated whether the number of colostrum feedings has any effect on health and performance of calves (Abuelo et al., 2021). Calves given a 2nd feeding of colostrum 6 – 12 h after birth were reported to have greater survival rates after encountering bovine respiratory disease or diarrhea compared to calves that received just one feeding of colostrum. In addition, the extra colostrum feeding increased preweaning ADG of heifers, and retained heifers provided the 2nd colostrum feeding tended to require fewer inseminations to become pregnant and tended to have greater first lactation milk production (Abuelo et al., 2021). Although these results are promising, more research is required to determine the impact of multiple colostrum feedings on calf health and performance.

Colostrum serves as a potential source for bacterial infection of calves, including exposure to *Mycoplasma paratuberculosis*, (Streeter et al., 1995; Sweeney, 1996) *Escherichia coli*, and *Salmonella* (Smith et al., 1989; Spier et al., 1991; Stabel et al., 2004). Previous published research studied whether colostrum could be pasteurized (63°C for 30 min), but pasteurization denatured immunoglobulins and caused the colostrum to thicken (Meylan et al., 1996; Godden et al., 2003). Heat-treating colostrum at 60°C for 60 min before administering it to calves has the potential to reduce pathogenic bacteria loads without denaturing immunoglobulins (Johnson et al., 2007; Elizondo-Salazar and Heinrichs, 2009a; Donahue et al., 2012). In addition, calves given heat-treated colostrum compared to raw milk had greater apparent efficiency of absorption, leading to greater serum total protein and IgG concentrations (Kryzer et al., 2015) from 24 h (Johnson et al., 2007) to 48 h after administration (Elizondo-Salazar and Heinrichs, 2009a), even up to 5 wk after colostrum administration (Elizondo and Heinrichs, 2009b). This increased transfer of passive immunity leads to reduced scouring events (Hesami et al., 2020) and disease treatments in calves administered heat-treated colostrum compared to calves administered raw colostrum (Godden et al., 2012). There is limited published data on the effect of administering heat-treated colostrum to calves on performance. Elizondo-Salazar and Heinrichs (2009b) found no differences in BW or feed intake between calves fed heat-treated or raw colostrum. Published evidence of increased ADG in calves fed heat-treated colostrum has been controversial (Rafiei et al., 2019), but researchers have consistently failed to observe improvements in feed conversion efficiency (Hesami et al., 2020). There is also no known published literature on the effect of heat-treated colostrum on productive performance later in life.

MILK ALLOWANCE

Standards for how much daily milk to allot calves have gradually increased over the years. Protocols that may restrict milk intake have led to concerns about calves being affected by periods of hunger, which may negatively impact health and performance. There is much variation in current industry practices on milk allowance, but on average calves are fed 4 – 5 L of milk per day (USDA, 2016). Rosenberger et al. (2017) tested calf intake and performance when whole milk was provided at 6, 8, 10, or 12 L per day. As expected, calves with higher milk allotments consumed more milk (Rosenberg et al., 2017; Jafari et al., 2020). However, calves fed 12 L/d did not consume starter before the 10-d weaning phase began on d 42, and even during the weaning phase consumed ≤ 50% as much starter as the 6 and 8 L/d treatments (Rosenberg et al., 2017). Despite the minimal starter intake for the 12 L/d treatment during the first 55 d, by the end of study on d 68 there was no overall difference among treatments for starter intake (Rosenberg et al., 2017). There was a linear increase in ADG with increased milk allowance over the course of the entire study (Jensen et al., 2015; Rosenberger et al., 2017). Other research found that calves fed greater amounts of milk have heavier body weights and increased feed efficiency (Jafari et al., 2020). There were no differences reported between calves fed 8 L/d (high) or 6 L/d (low) levels of milk on the incidence of diseases such as diarrhea and BRD (Bach et al., 2013). However, calves fed low levels of milk did have greater risk of BRD relapse after the first treatment (Bach et al., 2013). Surprisingly, a majority of the research published on milk allowance focuses on the social behavior of the calf and less on the performance and health (Jensen, 2003; Jenson and Holm, 2003; Krachun et al., 2010; Duve et al., 2012). More research is needed that focuses on indicators of performance and animal well-being with regard to milk allowance.

WEANING PHASE

AGE

Weaning is a pivotal time in a calf's life and a smooth transition from milk to solid feed is essential for growth and future performance. One of the factors that influences weaning success is the age at which the process is initiated. Early weaning is a method in which calves are weaned from milk within the first few weeks of life. An advantage of this approach is decreased labor and feed costs associated with calf rearing. Previous research indicated that early-weaned calves performed similarly to later weaned calves (Khoury et al., 1967; Winter, 1978; Winter, 1985). Winter (1985) weaned calves at either 3, 5, or 7 wk of age and found no differences in growth from birth to 14 wk of age. In addition, there were no difference among treatments in wk 9 to 14 for feed intake or feed efficiency. Calves weaned at 3 wk did show a reduction in N retention immediately post-weaning, but they returned to N accretion rates similar to those of other treatments by wk 8 to 14. This study established that early weaned calves could develop functional rumens and survive on solid diets by 3 wk of age.

More recent research has shown that there are some potential negative effects of early weaning, particularly when it comes to calf health (Hulbert et al., 2011). Hulbert et al. (2011) analyzed the innate immune system of calves weaned at 4 wk of life versus calves weaned at 7 wk. Weaning, regardless of age, was found to suppress innate immune function. Older calves had reduced cortisol and haptoglobin concentrations from 45 to 66 d of age, suggesting that these older calves were less stressed. In addition, early weaned calves had less TNF-α secretion in LPS-stimulated whole blood. Even though findings pointed to a suppressed innate immune system in early weaned calves, the authors discussed that there was potential for more

immunological resistance to disease in early weaned calves due to immunoglobulins passively derived from colostrum (Hulbert et al., 2011).

Eckert et al. (2015) used a 7-d weaning process initiated at two different ages (6 wk vs. 8 wk of age). Calves weaned at 6 wk of age had less ME intake from the transition period through 2 wk post weaning compared to calves weaned at 8 wk. This lesser energy intake resulted in less ADG for calves weaned at 6 wk during the same time period. Moreover, calves weaned later had greater starter intake for 2 wk before the weaning period, during the weaning period, and two weeks post-weaning. Conversely, more recent research showed no significant differences in ME intake or starter consumption between calves weaned at 60 vs. 75 d of age (Mirzaei et al., 2018). However, calves weaned at 75 d tended ($P = 0.06$) to have greater ADG for the entire study (Mirzaei et al., 2018). The increased ADG resulted in greater final BW and withers height and a tendency ($P = 0.09$) for greater body length in the calves weaned later. Weaning at 6-8 wk rather than at 4-5 wk of age, though adding significant labor and feed costs, can allow for more rapid growth early in life and may improve immune status of claves.

STARTER INTAKE

A commonly used rule of thumb in the dairy industry is that calves should be consuming approximately 1 kg of calf starter before weaning begins. This is not achieved across all individual calves, and the proportion of calves failing to achieve adequate starter intake prior to weaning likely varies by age at weaning. To better understand the implications of this variation, Benetton et al. (2019) recently investigated responses to weaning based on age (70 d, n = 16) versus based on adequate starter intake (n = 16). No differences were detected in final weight (d 98) among treatments, but calves weaned at 70 d of age were numerically heavier. It was noted

that 6 calves from the starter-based weaning treatment who failed to reach the required intake levels in order to be weaned during the study. These 6 animals were separated into their own cohort and analyzed accordingly (failed intake, n = 6; starter intake, n = 10). These calves had lighter final BW than age-weaned calves and tended to be lighter than calves that successfully reached the required intake levels. Calves that were successful in reaching the desired intake levels began weaning at approximately 52 d of age, which is almost 3 wk earlier than the conventional age-weaned calves (Benetton et al., 2019). Starter intake has shown to be an important factor in determining time of weaning. By waiting for starter intake to reach a certain threshold we are ensuring calves have the gastrointestinal development in order to effectively transition to only eating solid feed.

WEANING METHOD

There is quite a bit of variation in weaning methods within the dairy industry. Practicalities of labor cost, availability of labor, and facility design are often major factors influencing the duration of the weaning phase in calf rearing facilities. Sweeney et al. (2010) assigned 40 calves to 4 different weaning periods: 0, 4, 10, and 22 d. The study was designed so all calves were weaned by 41 d of age. Calves that began weaning at 19 d of age had lesser milk intake than the other treatments. In addition, calves in the 22-d weaning program had lesser ADG and BW at weaning. Conversely, calves that had a 0-d weaning period had lesser starter intake post-weaning than other treatments and actually lost weight over the 10 d post-weaning (Sweeney et al., 2010). An argument against the validity of this study is that, even though they gave the calves a 22-d weaning period, they started them on it when they were only 19 d old. As previously discussed, calves do not have a functional rumen capable of supporting a calf's

dietary needs within the first 3 wk of life. Therefore, even though the weaning period was longer and more gradual, the calves in the 22-d treatment simply did not have the anatomical maturity to compete with calves that began a weaning period at older ages. More recent research has found that an abrupt weaning over 1 d tended to result in greater BW at d 70 compared to a 21-d weaning period, with no other differences detected during the study (Bittar et al., 2020). The study consisted of 36 Holstein calves assigned to either of 2 weaning programs, with all calves weaned by 56 d of age. The most likely cause for differences in outcomes between the 2 studies is that age at weaning plays a larger role in weaning program success than the actual length of the weaning period.

It is possible that the number milk reduction steps in a weaning program could have an effect on calves. Klopp et al. (2019) studied the impact of a 2-step weaning program over 14 d compared to a 1-step weaning program over 7 d. No differences were found between the two weaning methods, except calves in the 2-step program had greater starter intake and hip height (Klopp et al., 2019). Even when the weaning period is the same length, the number of step-downs does not appear to have a major impact on performance (Parson et al., 2021). Within a 10-d weaning period, calves were assigned to either have 2 or 4 step-downs in milk allotment before being completely weaned. During the weaning period, calves on the 4-step program had greater DMI, but had lower DMI postweaning than calves on the 2-step weaning program. No other differences in performance were detected (Parson et al., 2021). Weaning method varies among operations within the industry. Research has indicated that age of weaning may play a more prominent role in calf performance than weaning length, but there is little research looking into the effect of abrupt weaning methods on calf and intestinal health. This area needs to be explored

so an optimal compromise between performance and health can be reached in the weaning process.

NON-NUTRITIONAL CONSIDERATIONS

AUTOMATIC FEEDERS

Automatic milk feeders are becoming increasingly popular tools to feed and manage calves while reducing labor costs on dairies. In addition to the reduction of labor costs, automatic feeders provide a tool for better monitoring of a calf's intake, growth, and health. This ability to more closely observe individual animal performance has influenced calf growers' ability to manage calves. de Passillé and Rushen (2012) evaluated the process of weaning 60 Holstein heifer calves based on starter intake. Depending on treatment, calves began weaning when their voluntary starter intake reached either 400 g/d or 200 g/d, and weaning ended when starter intake reached 1,600 g/d or 800 g/d. There was great variation in age of calves at the start of the weaning period (22 to 74 d). Though not significant, calves who started weaning at 200 g/d of starter intake tended to be younger than those assigned to begin weaning at 400 g/d starter intake. The authors did note that 11 of the calves on trial (20%) did not reach their starter intake goals and were nonetheless weaned at 74 d of age. Calves which started weaning at 200 g/d completed weaning at a younger age regardless of their ending weaning intake treatment; however, the authors noted that there was substantial variation in the data. Automatic feeders are a relatively new input to the calf rearing process but are becoming increasingly popular. Research on how best to utilize them in commercial practice is constantly competing with innovations in automatic calf feeder capabilities. As this technology develops we need to continue to study calf growth and health in regards to their use to see how they best fit in the industry.

HOUSING

It is a challenge to house calves in an environment that limits their exposure to pathogens while balancing their need to express social behaviors. Both factors influence calf growth and performance. Housing calves individually versus in pairs does not influence incidence of diarrhea (Chua et al., 2002; Pempek et al., 2016; Bučková et al., 2021) or BRD (Jenson and Larson, 2014; Bolt et al., 2017). In terms of growth and performance, there is discrepancy among studies regarding whether housing type effects intake and growth (Chua et al., 2002; Pempek et al., 2013; Bolt et al., 2017; Bučková et al., 2021, vs, Costa et al., 2015; Jensen et al., 2015; Pempek et al., 2016; Whalin et al., 2018). Studies that did report differences between individual versus pair-housed calves stated that pair-housed calves had greater growth and attributed this to calves learning from each other and perceived competition for food (Costa et al., 2015; Jensen et al., 2015; Pempek et al., 2016). Group housing calves shows potential to offer improved performance without sacrificing animal health compared to individually housed calves.

CONCLUSIONS

The first few months of life are the most critical for a newborn calf. Calves are exposed to several stressors which, if not managed properly, can have lasting effects on a calf's health and performance throughout its life. The health and nutrition of the dam have been shown to have lasting effects on the calf. Once the calf is born, it is imperative that the calf receive sufficient amounts of high-quality colostrum. To ensure this is executed properly, great care needs to be taken in handling and preparing the colostrum to ensure it is free of pathogenic bacteria and has adequate immunoglobulins. This will allow successful passive transfer to occur, which in turn leads to a healthier, better-performing calf. The weaning phase is another point of

stress in a young calf's life. Calves need to be old enough to consume sufficient quantities of calf starter before weaning; having a functioning rumen that can support nutrient requirements makes the weaning process easier. More research is needed to fully understand the effects of weaning on calf health and performance, so that dairy producers can determine the best balance between labor, input costs, and infrastructure with calf performance and health.

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Case Study: Combined risk factors and digestive disorders in mid-lactation Holstein cows

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INTRODUCTION

Digestive disorders sometimes occur among lactating cows on dairy farms. These sporadically occurring challenges, ranging from mild diarrhea to hemorrhagic bowel syndrome (Owaki et al., 2015), rarely offer an obvious cause, leaving producers confounded. Digestive challenges are believed to be multifaceted in their development (Kirkpatrick et al., 2001). Opportunistic gastrointestinal pathogens (Godden et al., 2001; Dennison et al., 2002), excessive flow of fermentable carbohydrate or protein to the hind gut (Gressley et al., 2011), and the presence of mycotoxins (Grenier and Applegate, 2003) in the diet are just some of the known sources of digestive disorders. These conditions are thought to contribute to a disruption of the gut barrier (i.e., "leaky gut"; Kvidera et al., 2017), which in turn leads to bacterial invasion and dramatic inflammation of the tissue (Godden et al., 2001; Kirkpatrick et al., 2001; Dennison et al., 2002). Previous research into this topic has been limited, as studies have assessed individual risk factors which have been inadequate to replicate the digestive disorders on their own (Ewolt et al., 2005; Gressley et al., 2016).

Gastrointestinal disease on commercial dairy operations may typically result from multiple risk factors occurring simultaneously. Documentation of these factors, throughout the course of an outbreak, would help to improve our understanding of the disease process, but commercial farms rarely obtain individual cow data or longitudinal samples which would allow for analysis of contributing factors prior to disease onset.

In the process of conducting a controlled nutrition study, we observed the onset and resolution of gastrointestinal disease. For experimental purposes, we had collected feed, milk, blood plasma, and fecal samples that have allowed us to document some relevant changes that

likely contributed to disease progression and resolution over the course of 14 weeks. Our objective in this case study is to describe our observations and evaluate plausible explanations for the disease process.

MATERIALS AND METHODS

All protocols were approved by the Kansas State University Animal Care and Use Committee. Fifteen multiparous (10 in lactation 2 and 5 in lactation 3+) Holstein dairy cows (94–197 days in milk; DIM) were part of a larger production study; here we report the temporal responses of control animals. Cows were housed at the Kansas State University Dairy Teaching and Research Center (Manhattan, KS) in tie-stalls with rubber mats and wood shavings. They were fed a total mixed ration (TMR; Table 2.1) twice daily, consisting of corn silage, alfalfa hay, corn byproduct (Sweet Bran, Cargill, Blair, NE), whole cottonseed, and a grain mix. Kansas experienced drought conditions during the summer of 2018, which resulted in the corn silage having low starch content and greater than normal NDF digestibility (Table 2.2). The low energy content of silage required greater concentrate levels to meet predicted energy requirements for highly productive mid-lactation cows. Feed and feed refusal samples were collected 3 times per week, combined into bi-weekly composites, and sent to Cumberland Valley Analytical Services (Madison, Wisconsin) for chemical composition analysis. Dry matter intake (DMI) was determined by recording daily feed intake and factoring in TMR dry matter content, updated weekly.

Cows were milked 3 times daily at 04:00, 11:00, and 18:00 h. Milk yield was recorded electronically at each milking. Milk samples were collected for 6 consecutive milkings each week and sent to Miner Institute (Chazy, New York) for component analysis. After analysis, data were composited by week. Energy corrected milk (ECM) yield was calculated as (0.327 × milk

yield) + (12.95 × fat yield) + (7.65 × protein yield) and feed efficiency was calculated as ECM/DMI.

Blood samples were collected from the coccygeal vein once every other week at 15:00 h into 2 evacuated 10-mL tubes containing potassium EDTA (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ). Blood was put on ice immediately after collection and then centrifuged at 2,415 × *g* (Beckman J06B Centrifuge; Brea, California, USA) for 15 min. Plasma was collected and stored at -20°C for later analysis. Plasma was analyzed for haptoglobin (Cooke and Arthington, 2012), D-lactate (Colorimetric Assay Kit; Biovision Inc., Milpitas, CA), serum amyloid A (SAA; ELISA Assay kit; Tridelta Development Ltd., Greystones Co., Wicklow, Ireland), lipopolysaccharide binding protein (LBP; ELISA Assay Kit; Cell Sciences Inc., Canton, MA; Bannerman et al., 2003), and diamine oxidase (DAO; Biovision Inc., Milpitas, CA).

Over the course of the study, 5 of the 15 cows were removed from the study for health concerns. Some loose manure was noted relatively early in the study, particularly from wk 4 on; however, for most cows and most days, manure consistency was within the normal range.

The first cow to be removed from study was in her 4th lactation and developed hock inflammation in wk 4. The next three cows were removed from the study for digestive disorders. During wk 5, the first serious summer heat stress window occurred, with mean weekly environmental temperature-humidity index (THI) climbing past 70 (Figure 2-1). At the end of wk 5, a second-lactation cow suddenly (within 24 h) went off feed and had a sharp decrease in milk production (from a mean of 38 kg/d down to 19.5 kg/d). Physical examination revealed extremely high rumen motility, but her body temperature and water intake were normal. Within 48 h, she was diagnosed with a displaced abomasum. In wk 7, two more second-lactation cows were removed from the study within 48 h of each other, after 4 d of declining feed intake.

Digestive tract abnormalities (high motility, diarrhea) were again observed, and one of these cows also showed some apparent neurological problems.

At this point in the study (wk 7), samples were sent for initial mycotoxin analysis (Romer Labs Inc. Union, MO, USA; LC-MS) and the diet was adjusted to partially replace some excessively dry corn silage (Corn silage A) with a more typical corn silage (Corn Silage B) and some straw to enhance physically effective fiber content of the diet. Furthermore, we began treating the approximately 2.4 m silage faces with 2 L/d of organic acids (Ultra-Curb, Kemin, Des Moines, IA) to limit mold growth at feed-out and added an anti-mycotoxin product (Biofix Plus Pro; Biomin America, Overland Park, KS) to the grain mix at 0.10% of the ration (DM basis). All of these changes were in place by the end of wk 8. One additional cow (third lactation) was removed from study during wk 13 due to clinical mastitis. Although we no longer sampled cows that were removed from the study, all cows recovered and remained in the herd.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Time series data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC). Data were modeled with the Mixed Procedure using week, parity group (2 vs. 3+), and week × parity group interaction as fixed effects with cow as a random effect. Repeated measures over time were modeled with a heterogenous autoregression covariance structure. Conditional Studentized residuals were used to check for normality and to remove outliers (> 4 or < -4). Significant effects were declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. All data are expressed as least squares means and standard errors of the means. Differences between wk 1 and subsequent weeks were evaluated by the pdiff option of the Mixed Procedure when the overall effect of week was significant.

RESULTS AND DISCUSSION

Dry matter intake and milk yield (Figure 2-2) were greater for cows in parity 3+ vs. parity 2 ($P = 0.01$) and both varied by week ($P < 0.01$) regardless of parity. Dry matter intake increased in wk 2 and 4 relative to wk 1 ($P < 0.05$), whereas milk yield was greater in wk 2 and 3, followed by lower production in wk 9, 10, 11, 12, 13, and 14 relative to week 1 ($P < 0.05$). Parity groups did not differ in milk fat content ($P = 0.47$, Figure 2-2C); however, there was week-to-week variation ($P = 0.01$). Fat content was decreased in wk 2, 3, 4, 5, 7, 9, and 10 relative to wk 1 ($P < 0.05$) before recovering in wk 11–14. Second lactation cows tended to have greater milk protein content compared with older cows ($P = 0.098$, Figure 2-2D); in addition, wk 2, 4, 7, 8, 10, 11, 13, and 14 all differed from week 1 ($P < 0.05$). Energy-corrected milk yield was greater in parity 3+ cows vs. second lactation cows ($P = 0.02$; Figure 2-2E), and wk 2, 9, 10, 11, 12, 13, and 14 differed from wk 1 ($P < 0.05$). Feed efficiency (defined as ECM yield / DMI) is shown in Figure 2-2F. Cows in lactation 3+ had greater efficiency than lactation 2 cows ($P = 0.01$), and efficiency was decreased in wk 2 and 4 vs. wk 1 ($P < 0.05$).

As a case study, interpretation of these observations must be carried out cautiously. Multiple factors were changing simultaneously during the study, including weather conditions, microbial and mycotoxin contaminants, forage sources, and mitigation strategies. It is notable that all 3 of the cows that left the study due to digestive disorders were second-lactation cows.

The uptick in feed consumption through wk 4 likely reflected adaptation to the new diet, whereas variation after that time likely reflect a combination of responses to heat stress and advancing stage of lactation. Although a progressive decrease in milk yield is expected in this group of cows past peak lactation (DePeters et al., 1985), the more dramatic decline in milk yield from wk 9 through 14 was likely in part a response to the change in diet, particularly the inclusion of dietary straw to increase effective fiber content. Previous research has shown that

adding slowly fermenting fiber to lactation diets typically decreases DMI and fluid milk yield (Chun Li et al., 2020).

It is well established that there is a negative impact of heat stress on feed intake and production in ruminants (Ominski et al., 2002; West, 2003; Allen et al., 2015; Polsky and von Keyserlingk, 2017). The THI threshold for negative impacts on a dairy cow is around 70 (St. Pierre et al., 2003). Throughout the study, mean ambient THI levels were consistently close to or above this threshold (Figure 2-1); although the barn housing the cows employed evaporative cooling to combat heat stress, it was not completely prevented during the heat of the day for most days after wk 4. Kadzere et al. (2002) demonstrated that there is a correlation between milk production and metabolic heat load, and recent research has postulated that the THI threshold for heat stress needs to be modified to match individual animal traits such as genetics and milk production (Sanchez et al., 2009; Hammami et al., 2015).

Mycotoxin concentrations detectable in the TMR and ingredients throughout the study are presented in Table 2.3 and Figure 2-3. Trichothecene concentrations in the diet stayed between 1327 ppb and 1735 ppb throughout the study. Zearalenone concentrations in TMR samples hovered around the detection limit of 81.4 ppb (dry-matter basis), with a peak concentration of 171.4 ppb detected. There is relatively little evidence available to establish concentrations of mycotoxins that are of concern for ruminants. There is some information regarding responses to aflatoxins (Queiroz et al., 2012; Ogunade et al., 2016; Ogunade et al., 2018; Jiang et al., 2018; Pate and Cardoso et al., 2018), but we did not detect that class in any ration sample analyzed. *Fusarium* fungi produce trichothecenes including nivalenol and deoxynivalenol (DON, also known as vomitoxin), the two compounds detected in our samples. Zearalenone is also produced by *Fusarium* fungi, but is an estrogenic metabolite. On their own,

these mycotoxins present challenges to producers, but previous research has shown that exposure to mixtures of mycotoxins can have a more acute impact (Alassane-Kpembi et al., 2017; Feijo Correa et al., 2018; Yang et al., 2020).

There is little information on feed-borne mycotoxins and their effect on ruminant production in a commercial setting. Driehus et al. (2008) surveyed and sampled 24 different dairies and found a wide range of mycotoxin concentrations in feed, with a majority of samples below concentrations usually considered to be concerning. Estimates of the daily mycotoxin intake came from surveying dairy managers, so it was hard to gauge actual mycotoxin exposure. Korosteleva et al. (2007; 2009) fed dairy cows TMR naturally contaminated with mycotoxins for extended periods of time (56 and 63 d, respectively). Neither study saw any effect of mycotoxins on production, even though they were feeding mycotoxins at a rate approximately 3 times that detected in the present study. Korostelelva et al. (2007) noted a depression of circulating immunoglobulin A, which the authors attributed to mycotoxin suppression of the immune system. Such immunosuppression could make the cow more susceptible to other challenges.

Blood samples collected every 2 weeks throughout the study were analyzed for haptoglobin, an acute phase protein that is elevated during systemic inflammation (Jacobson et al., 2004; Vels et al., 2009). Plasma haptoglobin concentrations (Figure 2-4A) tended to be greater in second lactation cows vs. parity 3+ cows ($P = 0.08$), and wk 1 differed from wk 3, 5, 9, and 11 ($P < 0.05$). The temporal pattern aligned with visual observations of digestive dysfunction (e.g., excessive gut motility, loose fecal consistency) and suggested that changes put in place in weeks 7–8 likely had a positive impact on the inflammatory status of cows by week 11. Previous research has established that haptoglobin increases with heat stress in ruminants (Hamzaoui et al., 2013; Alberghina et al., 2013; Leiva et al., 2017). However, trichothecene

mycotoxins have not altered haptoglobin concentrations in previous studies in lactating dairy cattle (Korosteleva et al. 2007; 2009). Furthermore, haptoglobin results varied in swine fed mycotoxins (Swamy et al., 2003; Díaz-Llano and Smith, 2007; Wu et al., 2015). Wu et al. (2015) reported an increase in serum haptoglobin concentrations as concentration of mycotoxins increased in diets fed to growing pigs. However, Díaz-Llano and Smith (2007) reported no difference in haptoglobin concentrations of first-parity sows fed mycotoxin infected feed compared to negative controls. As previously discussed, it is possible that the mycotoxins made the cows in the present study more susceptible to heat stress and other forms of stress, potentially exacerbating inflammatory responses.

D-lactate concentrations (Figure 2-4B) in plasma tended ($P = 0.051$) to be greater in second lactation cows compared to parity 3+ cows, and wk 7, 11, and 13 differed from week 1 ($P < 0.05$). D-lactate is produced primarily by microbial metabolism; its increased concentration in plasma by wk 7 likely indicates a decline in gut barrier function (leaky gut) and/or excessive hind-gut fermentation, with an apparent return to a more normal status by the end of the study. Harmon et al. (1985) found increased blood D-lactate concentration in steers with induced acute acidosis compared to negative controls. Elevated D-lactate blood concentrations have been associated with acidosis and clinical signs of illness (diarrhea, behavior, eating habits, etc.) in calves (Lorenz, 2004). Furthermore, D-lactic acid found in blood is most likely produced in the hindgut from starch that has bypassed the rumen (Lorenz, 2009). Previous research has concluded there is a greater correlation between hindgut D-lactate production and serum D-lactate concentrations than D-lactate produced in the rumen (Grude, 1999; Lorenz, 2004; Ewaschuk et al., 2004). In the first 5 weeks of the study there were significant decreases in milk

fat concentrations (Figure 2-2C), which likely reflects some of the same changes to the microbial ecosystem that drove increased D-lactate production.

Diamine oxidase is a digestive enzyme produced by epithelial cells in the intestine, and lysed enterocytes are thought to be the primary source of DAO in the blood stream (Wollin et al., 1998). Plasma DAO was therefore evaluated as a potential proxy for intestinal enterocyte mass. Diamine oxidase activity (Figure 2-4C) was greater in parity 2 versus parity 3+ cows ($P = 0.01$). Plasma DAO activity was decreased in weeks 5 – 13 compared to week 1 (all $P < 0.05$), independent of parity. As a relatively unexplored biomarker in plasma, interpretation of the DAO data is not straightforward (Celi et al., 2009); the concentration of the enzyme is a function of both enterocyte mass and the rate of enterocyte lysis. In our findings, though, plasma DAO activity was clearly reduced during the weeks when clinical signs pointed to gastrointestinal distress in these cows, coincident with markers of dysbiosis (D-lactate) and systemic inflammation (haptoglobin). In alignment with our findings, Fukuda et al. (2019) reported that plasma DAO concentration (determined by ELISA, not enzymatic assay) was reduced in pre-ruminant calves with diarrhea. These results suggest that plasma and mucosal DAO activity may both decrease during periods of intestinal disease in cattle.

We are not aware of published data on blood DAO activity among cattle fed mycotoxins, but pigs fed *Fusarium*-infected feed showed increased blood DAO (Ji et al., 2019). Furthermore, poultry fed *Fusarium*-infected feed for 42 d also had increased blood DAO compared to negative controls (Cheng et al., 2018). We speculate that compounds such as mycotoxins that act as stressors - but may not lead to overt intestinal disease - promote adaptive changes in the mucosa that enhance DAO activity in the tissue, and in turn, in plasma. We observed greater plasma DAO concentrations among 2nd-parity cows, the group that also had greater inflammatory

biomarker concentrations and clinical disease, although there was no evidence of a time × parity interaction. Currently there is limited published research on DAO as an intestinal health marker, and more research is required to validate its utility for investigating enteric health in cattle.

Lipopolysaccharide binding protein (Figure 2-4D) did not differ by parity ($P > 0.10$), but plasma LBP concentration was greater ($P = < 0.01$) in wk 11 and 13 than wk 1 ($P < 0.05$). Lipopolysaccharide (LPS) is an endotoxin produced by Gram-negative bacteria, which is released upon their lysis. Lipopolysaccharide binding protein binds LPS, limiting its immunogenic potency and enhancing its clearance from circulation (Schumann et al., 1990; Wright et al., 1990). As an acute-phase protein, increased circulating LBP concentrations indicate that more inflammatory agents, potentially including LPS and/or other signals, were reaching the liver and altering its function (Bradford et al., 2015). We are unable to explain the surprising differences in temporal patterns in haptoglobin vs. LBP, given that both are acute-phase proteins expected to be induced by similar stimuli. Clinical observations of disrupted health certainly aligned better with the time period of haptoglobin elevation rather than LBP in this study. Recent findings suggesting that the most important cellular source of LBP may be enterocytes, which release lipoproteins enriched in LBP to prevent hepatic inflammation after LPS translocation from the gut (Han et al., 2021). Considering this new information, peripheral concentrations of LBP may be less insightful than portal concentrations with respect to adaptive release of this acute phase protein.

Serum amyloid A is another acute phase protein that increases in concentration when an animal is experiencing an inflammatory response. We did not detect parity or time effects for SAA ($P > 0.10$). The lack of significant effects for SAA is interesting considering the results for other acute phase proteins, haptoglobin and LBP. Previous research has shown variation in SAA

concentrations during a mycotoxin challenge. Pate and Cardoso (2018) found no differences in SAA concentrations between lactating cows fed a diet infected with aflatoxin versus a negative control. A similar study (Pate et al., 2018) found no differences in SAA concentrations in lactating dairy cows fed aflatoxin-infected feed compared to negative controls. Both of these studies evaluated only aflatoxins, whereas a majority of the mycotoxins in the present study were trichothecenes. There is little controlled research on trichothecenes and how they impact dairy cows. Swine (n = 41) fed either a diet containing deoxynivalenol or a negative control diet for 4 wk showed no increase in SAA concentrations (Dänicke et al., 2020). According to our findings and previous research, mycotoxins may not affect SAA concentrations.

Twice during the study (wk 9 and 14), feed and fecal samples were collected to enumerate viable Clostridia bacteria and those from the species *C. perfringens* specifically. The drought-stressed dry corn silage had Clostridia concentrations in wk 14 almost 50× greater than samples collected in wk 9, and the concentration of Clostridia in the TMR increased between wk 9 and 14 (Table 2.4). In contrast, fecal samples showed decreased total Clostridia as well as *C. perfringens* between wk 9 and 14. There was therefore great disparity between the feed and fecal sample *C. perfringens* microbial concentrations (Table 2.4); even though feed *C. perfringens* concentrations increased in the second half of the study, fecal *C. perfringens* concentrations decreased. This suggests a shift in the gut microbial ecosystem independent of feed contamination. Change in diet composition or in mycotoxin-microbe-gut interactions following the incorporation of mitigation strategies could have allowed for a more robust microbial ecosystem able to prevent *C. perfringens* dominance (La Ragione and Woodward, 2003; Wang et al., 2017) Unfortunately, microbial samples of the gut were not collected during the study and therefore we cannot evaluate this hypothesis. *Clostridium perfringens* has been implicated in

several diseases within the ruminant animal, most notably jejunal hemorrhagic bowel syndrome (Elhanafy et al., 2013). However, previous research has shown that inoculating an animal with *C. perfringens* does not necessarily cause the disease (Abutarbush and Radostits, 2005). For this reason, is it considered an opportunistic pathogen which takes advantage when the host animal has suppressed immunity due to another stressor. None of the animals in this group experienced jejunal hemorrhagic bowel syndrome, but there is potential that the *C. perfringens* in the diet was an additional stress on the animals.

We propose that the gastrointestinal health challenges observed during this study emerged as a result of "stacked stressors." The diet formulation likely introduced one risk factor; although not extreme, the diet was marginal in terms of supply of physically effective fiber (Humer et al., 2018), which made disturbance of the gut microbial ecosystem more likely. Secondly, the consistent exposure to mycotoxins likely contributed to disruption of both the microbial populations and the gut itself (Park et al., 2015). Finally, the onset of summer heat stress seemed to tip some of the cows over the edge into clinical disease.

Assuming that the proposed etiology is correct, what could have been done to avoid these problems? Because we did not see a drop in the measurable mycotoxins (Figure 2-3) or culturable Clostridia (Table 2.4) in feed during the study, and heat stress continued to challenge the cows (Figure 2-1), the resolution of disease biomarkers and clinical signs of digestive problems by week 11–13 suggests that at least some of our changes were likely effective. Increasing dietary forage has a protective role in microbial stability in the gut (Lindberg, 2014), and directly contributes to slowing passage of feed through the gastrointestinal tract (Poore et al., 1990). Organic acid treatment of the silage face (particularly for an excessively dry silage) can help to limit fungal growth at feed-out (Kung et al., 2000; Driehuis et al., 2001), and mycotoxin

binding products are effective at binding at least some mycotoxins (Vila-Donat et al., 2018), helping to wash them out of the gut with less impact. Unfortunately, due to variable weather patterns in the United States mycotoxins are becoming more prevalent in animal feeding programs and need to be addressed (Magan et al., 2011; Wu et al., 2011; Hendel et al., 2020).

CONCLUSIONS

Few publications have reported temporal responses of lactating dairy cows through a spontaneous gastrointestinal health challenge. Parity 3+ cows had greater dry matter intake and milk production than 2nd parity cows, but 2nd parity cows had increased concentrations of inflammation and gut disease markers such as haptoglobin and LBP compared to parity 3+ cows. A combination of suboptimal diet quality issues (mycotoxin, pathogen load, and limited physically effective fiber), compounded with seasonal heat stress, likely combined to encourage growth of opportunistic pathogens in the hind gut and alter gastrointestinal function. We hope that these observations can inform the development of testable hypotheses that may lead to a more robust understanding of how stacked stressors promote gastrointestinal disease.

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FIGURES

Table 0.1. Ingredient and chemical composition of total mixed ration (% of dry matter except where noted).

	Weeks -2–5	Weeks 6–12
Ingredients		
Corn silage A	16.7	6.29
Corn silage B	-	6.29
Alfalfa hay	20.8	20.9
Corn milling product[1]	17.4	17.5
Cottonseed	2.79	2.81
Corn grain	24.8	25.0
Expeller soybean meal[2]	12.2	12.2
Limestone	1.25	1.26
Sodium bicarbonate	0.85	0.86
Calcium salts of long-chain fatty acids[3]	0.83	0.84
Micronutrient pre-mix[4]	2.54	2.44
Wheat straw	-	3.51
Direct-fed microbial[5]	-	0.11
Nutrients		
DM, % as-fed	61.7	59.9
Net energy for lactation, Mcal/kg DM	1.69	1.63
Crude protein	18.5	19.4
Ether extract	5.03	5.01
Neutral detergent fiber	31.4	31.3
Acid detergent fiber	20.3	20.9
Ash	8.46	9.72
Ca	1.00	1.25
P	0.49	0.50

[1]Sweet Bran, Cargill, Blair, NE.
[2]Soy Plus, Landus Cooperative, Ames, IA.
[3]Essentiom, Arm & Hammer Animal Nutrition, Princeton, NJ.
[4]MegaLacR, vitamin E, salt, trace mineral, magnesium oxide, zinpro 4 plex c, zinpro 120, Rumensin 90, AjiPro L, Methionine MHA
[5]Biofix Plus Pro; Biomin America, Overland Park, KS.

Table 0.2. Chemical composition of corn silage sources used (% Dry-matter basis)

	Corn silage A	Corn silage B
Component, % DM		
Dry matter[1]	42.5	29.1
Crude protein	9.15	10.7
ADF	25.6	26.0
aNDF	41.4	45.9
Lignin	2.71	2.6
Starch	28.1	18.4
Crude fat	3.2	3.6
Ash	5.5	6.3
Calcium	0.3	0.3
Phosphorus	0.2	0.3
Magnesium	0.2	0.2
Potassium	0.9	1.6

[1]As-fed basis

Table 0.3. Mycotoxin concentrations (mean ± SEM) of diet and ingredients[1]

	Nivalenol, ppb[2]		Deoxynivalenol, ppb[3]		Fumonisin B1, ppm[4]		Zearalenone, ppb[5]		Ochratoxin A, ppb[6]	
Weeks	1-7	8-14	1-7	8-14	1-7	8-14	1-7	8-14	1-7	8-14
Total Mixed Ration	688 ± 26.6	344 ± 102	305 ± 39.8	604 ±71.5	0.3 ± 0.0	0.7 ± 0.1	68.8 ± 4.0	89.5 ± 5.9	ND	ND
Ingredient										
Grain mix	ND	ND	203 ± 77.5	202 ± 57.8	3.6 ± 1.1	2.2 ± 0.2	192 ± 32.7	73.3 ± 2.5	ND	ND
Corn silage A	1409 ± 96	666 ± 115	ND	ND	0.3 ± 0.1	0.5 ± 0.02	ND	ND	2.5 ± 0.7	1.8 ± 0.8
Corn silage B	ND	1218 ± 9.0	ND	ND	ND	ND	ND	ND	ND	ND
Sweet bran	2367 ± 233	2501 ± 260	4999 ± 824	2386 ± 510	2.0 ± 0.2	2.0 ± 0.1	636 ± 7.7	564 ± 20.8	ND	ND
Alfalfa hay	162 ± 46.3	197 ± 25.6	ND	120 ± 0.0	ND	ND	ND	ND	ND	ND
Cottonseed	ND	ND	133 ± 0.0	155 ± 0.0	ND	ND	ND	ND	ND	ND
Wheat straw	ND	145 ± 15.9	ND	2159 ± 955	0.3 ± 0.0	0.1 ±0.1	ND	56.8 ± 1.8	ND	ND

[1]ND = Below detection limit
[2]Limit of detection = 100.0 ppb
[3]Limit of detection = 100.0 ppb
[4]Limit of detection = 0.1 ppm
[5]Limit of detection = 51.7 ppb
[6]Limit of detection = 1.1 ppb

Table 0.4. Average Clostridia and *C. perfringens* enumerated in feed and fecal samples

	Clostridia CFU[1]/g		*C. perfringens* CFU/g	
	Week 9	Week 14	Week 9	Week 14
Feed samples				
Total mixed ration	1,280	8,700	100	50
Corn silage A	440	21,850	20	50
Corn silage B	20	<10	<10	<10
Fecal samples	34,000	5,000	31,000	4,100

[1]Colony forming units; an approximation of viable bacteria.

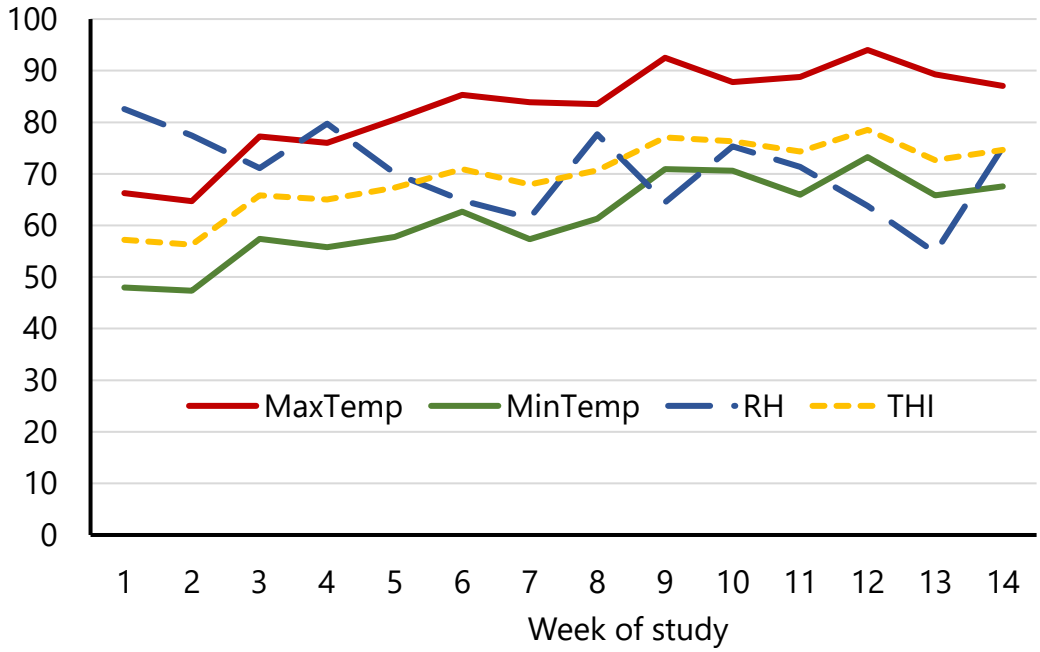

Figure 0-1. Mean external maximum daily temperature (MaxTemp, °F), minimum daily temperature (MinTemp, °F), relative humidity (RH, %), and temperature-humidity index (THI) by week of the study.

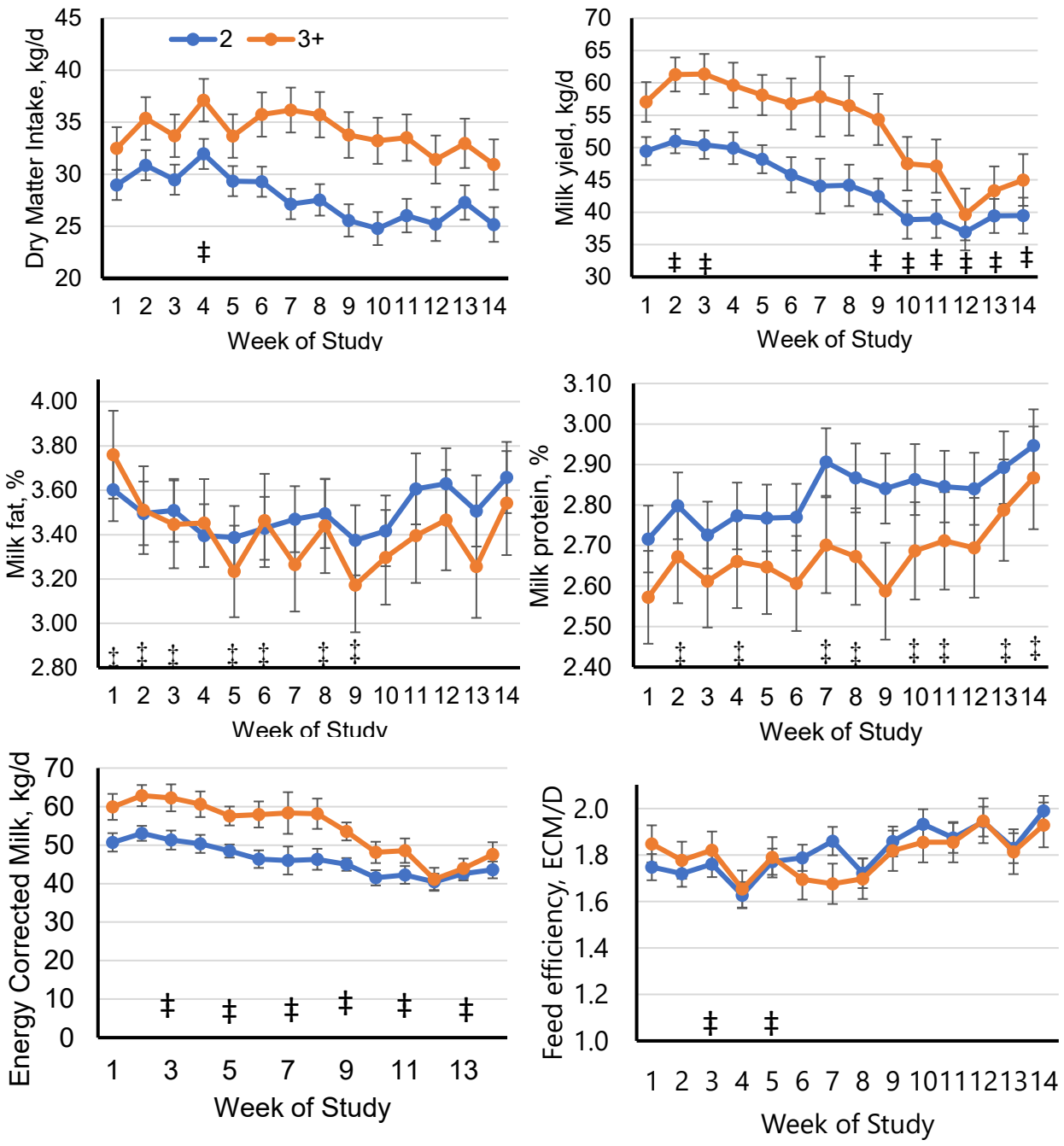

Figure 0-2. Productivity of cows through a spontaneous digestive disease outbreak. A) Dry matter intake; B) milk production; C) milk fat concentration; D) milk protein concentration; E) energy-corrected milk yield; F) feed efficiency. Values are means ± standard errors. ‡ $P < 0.05$ vs. week 1.

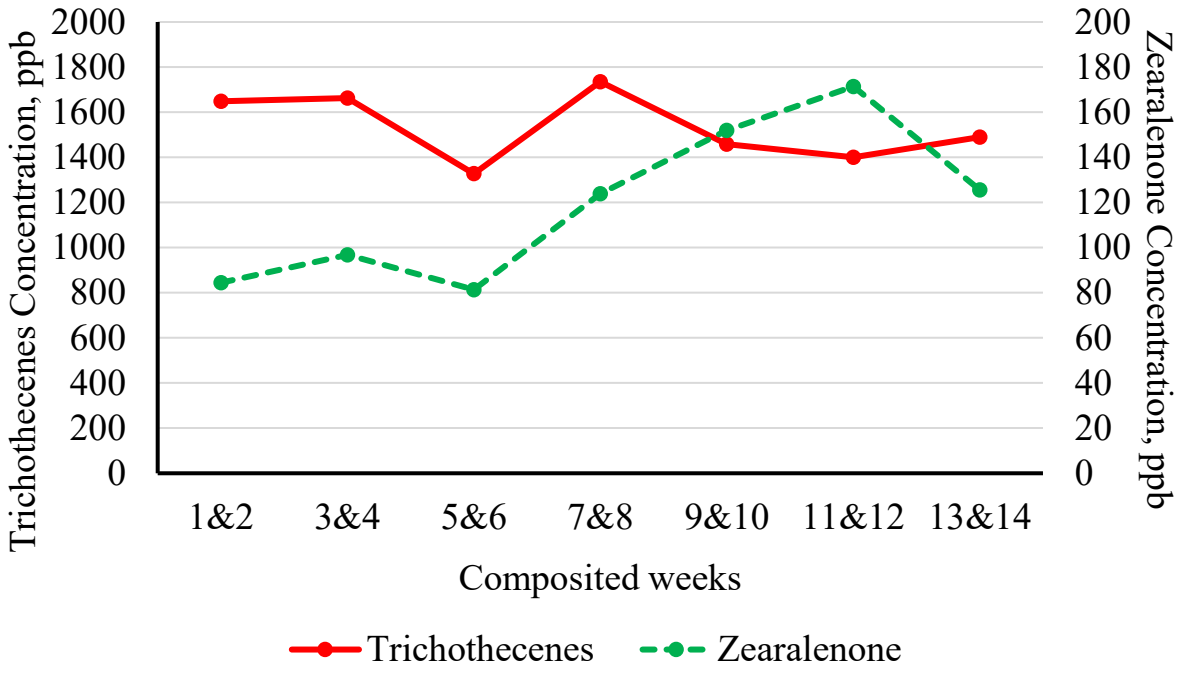

Figure 0-3. Mycotoxin concentration of composite ration samples (dry-matter basis). Values are means, with trichothecenes being the summation of nivalenol and deoxynivalenol concentrations. **Weeks where zearalenone concentrations were below detection limit of 84.1 ppb (DM basis).

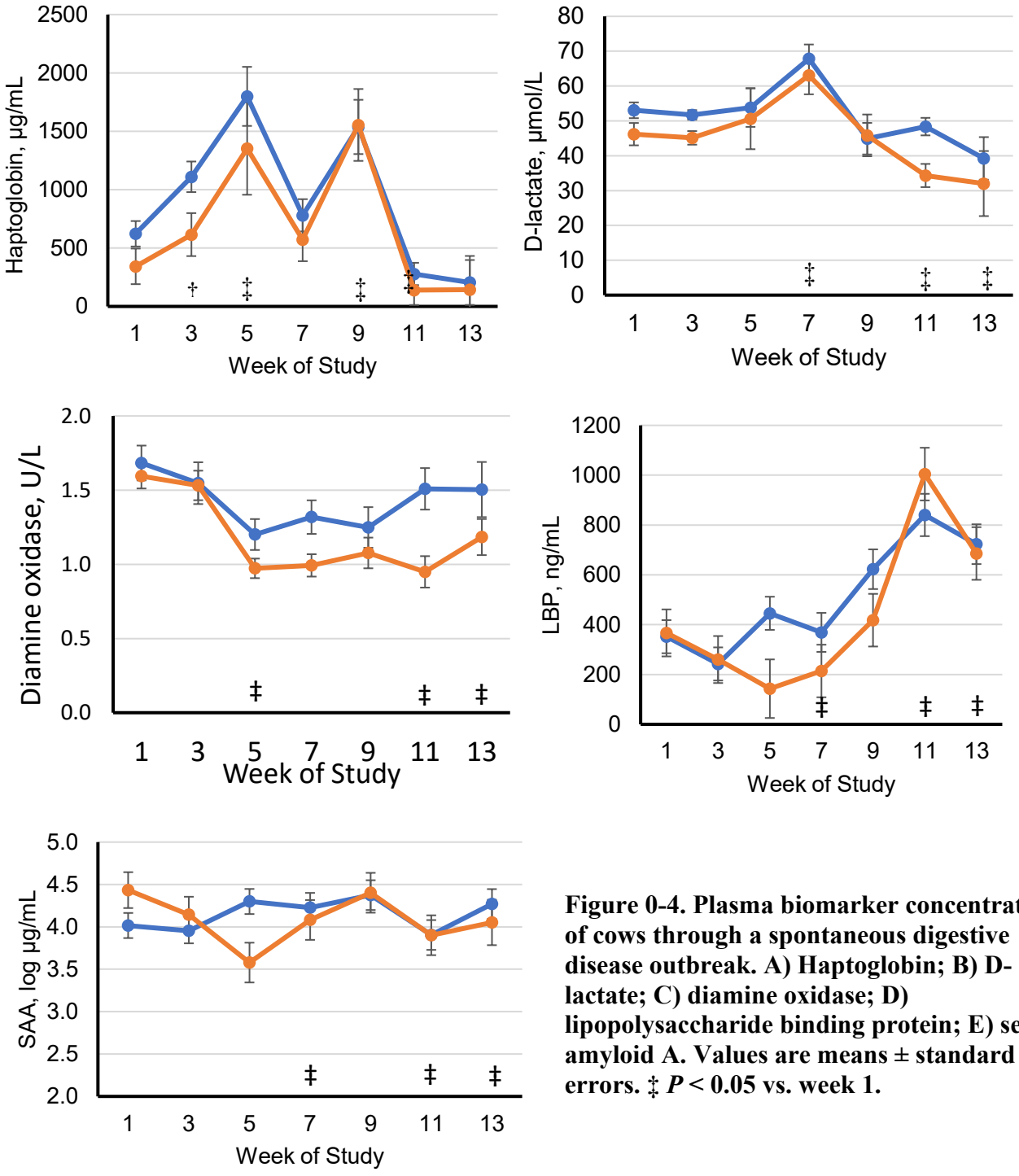

Figure 0-4. Plasma biomarker concentrations of cows through a spontaneous digestive disease outbreak. A) Haptoglobin; B) D-lactate; C) diamine oxidase; D) lipopolysaccharide binding protein; E) serum amyloid A. Values are means ± standard errors. ‡ $P < 0.05$ vs. week 1.

Chapter 3 -Effects of calcium gluconate supplementation on lactation performance and feed efficiency of mid-lactation Holstein cows

S. T. Quanz, L. K. Mamedova, K. E. Griswold, S. K. Kvidera, R. S. Fry, and B. J. Bradford

INTRODUCTION

Gluconic acid has been shown to increase acetate, lactate, and butyrate concentrations in cecal digesta of pigs (Tsukahara et al., 2002). This is accomplished through interactions with commensal bacteria in the cecum of pigs. Lactobacillaceae use gluconic acid as a substrate, converting it to acetic and lactic acids. Acetate and lactate in the cecum are subsequently used as substrates by *Megasphera elsdenii*, which produces butyrate (Tsukahara et al., 2002). Butyrate in ruminants can improve rumen epithelial growth (Penner et al., 2011), and similar impacts on colonocyte health have been reported in non-ruminant species. This impact on the gut epithelium is accomplished through cellular proliferation and inhibition of cell death. Unfortunately, the effects of butyrate and many other nutrients on health of the distal intestinal tract in ruminants is unclear due to limited research on the topic to date.

Calcium gluconate supplementation in ruminants is relatively new. Previous research (Doelman et al., 2019) has shown that infusing calcium gluconate into the abomasum of lactating dairy cows increased fecal butyrate concentrations. In addition, cows supplemented with calcium gluconate had greater milk fat concentration and tended to have increased fat yield (Doelman et al., 2019). These outcomes led to the development of a rumen-protected calcium gluconate designed to be used commercially. The impact on fat yield provides a potential economic driver for commercial supplementation of calcium gluconate in the dairy industry. However, more research is needed to validate these findings and to provide insight into mode of action.

The objective of this study was to evaluate the effect of dietary supplementation of fat-embedded calcium gluconate on lactation performance, feed efficiency, and biomarkers of gastrointestinal health in mid-lactation Holstein cows.

MATERIALS & METHODS

Thirty multiparous Holstein cows between 94 – 197 days in milk (DIM) were selected from the Kansas State University Dairy Teaching and Research Center herd. Cows were randomly assigned to tie stalls where they were fed the basal diet (Table 3.1) for a 14-d covariate period. Feed intake, milk production and composition, blood biomarkers, body weight (BW) and body condition score (BCS) were determined during the covariate period. Cows were milked 3 times a day and were allowed ad libitum feed and water. Five days before the treatment period, cows were blocked by parity, covariate period milk yield, BW and BCS, and randomly assigned to treatment within block in a randomized complete block design. Cows were fed the same basal diet formulated using NDS Software (RUM&N Sas, Reggio Emilia, Italy) and were given one of two top-dress treatments: 1) Control; NutraCor palm fat fed at 0.04% of diet dry matter, or 2) **eCaG** (Selko Cremalto; Trouw Nutrition USA, Highland, IL), a commercially available fat-embedded calcium gluconate, fed at 0.07% of diet dry matter.

Dietary forages, TMR, and feed refusal samples were collected 3 times per week, while diet concentrate samples were collected once weekly. Samples were dried in a forced-air oven at 105°C for 24 h to determine DM. Samples were combined into bi-weekly samples and sent to Cumberland Valley Analytical Services (CVAS; Madison, Wisconsin) for nutrient analysis using NIR. Dry matter intake (DMI) was determined by collecting daily feed intake and multiplying by TMR DM content.

Milk yield was recorded electronically at each milking. Milk samples were taken from 6 consecutive milkings and shipped to The Miner Institute (Chazy, New York) to be analyzed for fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), urea nitrogen (MUN spectrophotometer, Bentley Instruments), somatic cell count (SCC 500, Bentley

Instruments), and fatty acids using MIR analysis (Delta Combi 300; Drachten, Netherlands). After analysis, data were composited by week. Energy-corrected milk (ECM) was calculated as (0.327 × milk yield) + (12.95 × fat yield) + (7.65 × protein yield). Somatic cell score was determined using the equation: $\log_2(SCC/100) + 3$, according to Shook et al. (1993). Feed efficiency was calculated as ECM/DMI.

Cows were weighed once weekly immediately after the morning milking. Body condition scores were collected one day each week by 2 trained individuals scoring each cow independently on a 1 to 5 scale (Ferguson et al., 1994).

Blood was collected every other week (between the 2nd and 3rd milking) from the coccygeal vessels into 2 evacuated 10-mL tubes containing potassium EDTA. Immediately after collection, blood was put on ice until samples were centrifuged at 2,415 × *g* (Beckman J-6B Centrifuge; Brea, California, USA) for 15 min. Plasma was harvested and stored at -20°C until analysis. Plasma was analyzed for glucose (Kit No. 439-90901; Wako Chemicals USA Inc.), beta-hydroxybutyrate (BHB; Kit No. H7587-58; Pointe Scientific Inc., Canton MI), non-esterified fatty acids (FFA; NEFA-HR kit; Wako Chemicals USA Inc., Richmond, VA), and insulin (Kit No. 10-1201-01; Mercodia AB, Uppsala, Sweden). In addition, plasma was analyzed for gastrointestinal integrity and inflammatory biomarkers citrulline (Homocitrulline/Citrulline ELISA Assay Kit, No. ab242292; Abcam, Cambridge, MA), diamine oxidase (Fluorometric Assay Kit, No. K496; Biovision Inc., Milpitas, CA), D-lactate (colorimetric Assay Kit, No. K667; Biovision Inc., Milpitas, CA), and the acute phase proteins haptoglobin (Cooke and Arthington, 2012), serum amyloid A (ELISA Assay kit, No. TP-802; Tridelta Development Ltd., Greystones Co., Wicklow, Ireland), and lipopolysaccharide binding protein (Human LBP Multispecies Reactive ELISA Kit, No. CKH113; Cell Sciences Inc., Canton, MA).

Over the duration of the study, we experienced some animal health challenges which are detailed in Quanz et al. (2021). Briefly, 7 cows had to be removed throughout the course of the study for various reasons (Table 3.2). Four of those were removed for potential displaced abomasum (DA) of which 2 required surgery, 2 for lameness concerns, and 1 for mastitis. This reduced the number of cows completing the study to 23 (Control = 10, eCaG = 13). After finding evidence of mycotoxin contamination of several ration ingredients and heating of the TMR, beginning in week 5 of the treatment period, we initiated organic acid treatment (Ultra-Curb; Kemin, Des Moines, IA) of the silage face once daily and made several minor changes to the diet (Table 3.1; Quanz et al., 2021).

STATISTICAL ANALYSIS

Data from cows that were removed from the study were included in analysis up to the point where health or productivity deviations were noted. Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC). Data were modeled with the MIXED procedure of SAS with covariate data, treatment, week, and treatment × week as fixed effects and block as a random effect. Additionally, fixed effects of covariate × treatment were initially included in models, but if $P > 0.10$, they were removed from analysis. Repeated measures over time within cow were modeled with autoregressive [AR(1)] or heterogenous autoregressive [ARH(1)] covariance structures, selected based on the least Bayesian Information Criterion value. Conditional Studentized residuals were used to check for normality and to remove outliers (>4 or <-4). Treatment effects were declared at $P \leq 0.05$ and tendencies for treatment effects at $0.05 < P \leq 0.10$. All data are expressed as least squares means and standard errors of the means.

RESULTS

Production and efficiency data are shown in Table 3.3. We observed no treatment effects for DMI (Figure 3-1A), milk yield (Figure 3-1B), BCS, or BW. Total milk fat concentration tended to be greater ($P = 0.056$; Figure 3-1C), whereas protein ($P = 0.08$; Figure 3-1D) and lactose ($P = 0.07$) concentrations tended to be decreased for eCaG-supplemented cows (Table 3.3). No impacts on fat or protein yields were detected. Overall, eCaG had no effect on ECM ($P = 0.20$) or feed efficiency ($P = 0.16$).

The percentages of *de novo* synthesized fatty acids ($P = 0.02$) and mixed-source fatty acids in milk were greater ($P < 0.01$) for eCaG supplemented cows, while the percentage of preformed milk fatty acids was greater ($P < 0.01$) in Control cows. Furthermore, yields of *de novo* fatty acids ($P = 0.04$) and mixed fatty acids ($P = 0.05$) were greater for eCaG-supplemented cows. There were covariate × treatment interactions for proportions of preformed ($P = 0.02$; Figure 3-2A) and *de novo* synthesized ($P = 0.03$; Figure 3-2B) fatty acids. Both of these interactions demonstrate a tighter relationship between pre-study *de novo* and mixed FA proportions with treatment period proportions for eCaG-supplemented cows (regression coefficients closer to 1). Control cows had flatter slopes for these regressions, meaning that cows with relatively lesser proportions of *de novo* and mixed FA prior to the treatment period had an increase in these proportions, whereas Control cows that started with greater proportions of *de novo* and mixed FA prior to the treatment period showed a decrease in these proportions.

Effects of treatment on blood metabolites are shown in Table 3.4, and treatment × time means are plotted in Figure 3-3. Supplementation of eCaG had no overall effect on plasma citrulline, SAA, BHB, haptoglobin, D-lactate, LBP, glucose, or insulin concentrations, nor on DAO activity. Cows supplemented with eCaG had increased plasma free FA ($P = 0.04$). Furthermore, DAO responses interacted with pre-trial covariate values (Figure 3-2C). This

interaction showed that pre-study DAO had almost no relationship with treatment period DAO for Control cows, but had a nearly linear relationship for eCaG-supplemented cows. Finally, a tendency for a treatment × week interaction was found for LBP (P = 0.09; Figure 3-3F), driven by a decrease in LBP for eCaG in week 9 (P = 0.04).

DISCUSSION

Doelman et al. (2019) infused different concentrations (0, 44, 93, 140, or 187 g/d) of CaG into the abomasum of 6 lactating cows to assess effects on fecal VFAs, blood metabolites, and milk production. Increasing doses of CaG resulted in a quadratic effect in fecal isobutyrate with concentrations peaking at 44 g/d of supplemental CaG. Furthermore, plasma glucose and FFA concentrations decreased while BHB increased in CaG infused cows. From a production standpoint, cows infused with CaG had greater milk fat concentration and tended to produce more milk fat and ECM (Doelman et al., 2019). These results led to the two experiments reported by McKnight et al. (2019). In experiment 1, a 5 × 5 Latin square using 5 lactating cows and 5 treatments (0, 5, 17, 32, or 46 g/d of CaG), cows infused with CaG had decreased DMI and greater milk fat production. In experiment 2, 30 lactating cows were used in a cross over study design comparing a negative control to 46 g/d of CaG delivered orally. Cows supplemented CaG had lesser milk, milk protein, and lactose yields than those on the control treatment. In addition, cows fed CaG had reduced ruminal propionate concentration (McKnight et al., 2019).

Seymour et al. (2021) next took supplementation of CaG a step closer to commercialization. Forty-five lactating cows were used in a 3 × 3 Latin square design consisting to evaluate 3 treatments: a negative control mash, a mash containing CaG, and an extruded pellet containing CaG. Cows supplemented with either CaG tended to have greater fat-corrected milk (FCM), ECM, and fat yields. In addition, CaG-supplemented cows had greater FFA

concentrations and tended to have greater concentrations of preformed fatty acids in their milk compared to the control (Seymour et al., 2021).

In the current report, we found no impacts of treatment on overall milk component production or production efficiency. We did observe tendencies for increased milk fat concentration and decreased milk protein and lactose concentrations. Furthermore, supplementation of eCaG caused an increase in milk de novo fatty acid production. De novo fatty acids are so called because they are newly formed in the mammary gland, predominantly utilizing acetate and butyrate (Palmquist, 2006). These VFA are typically formed through the microbial breakdown and fermentation of fibrous feeds (Palmquist et al., 1993).

Previous research has studied the effect of supplementing gluconic acid in pig cecal digesta and reported an increase in butyrate production cause by lactic acid producing bacteria (LAB; Tsukahara et al., 2002). Gluconic acid serves as a substrate for LAB which produce lactic acid that serves as a substrate for lactic acid consuming bacteria such as *Megaspheara elsdenii*, which metabolizes it to produce butyrate and propionate. *Megaspheara elsdenii* has also been reported to ferment gluconic acid directly into acetate, formate, and n-butyrate (Tsukahara et al., 2002). Tsukahara et al. (2002) and Biagi et al. (2006) analyzed the effect of gluconic acid supplementation on VFA production in cecal fluid from pigs and observed increases in acetate, butyrate, and propionate concentrations compared to a control.

Assuming the eCaG completely bypassed the rumen, it would have been available in the hindgut to potentially influence fermentation patterns, with impacts likely to be similar to those described above. Assuming butyrate production was increased, butyrate could be absorbed through the intestinal wall and enter the animal's blood stream as butyrate or partially metabolized to BHB (Weigand et al., 1975). The additional butyrate would then be available as a

substrate for de novo fatty acid production in the mammary gland, potentially increasing de novo milk fatty acids as observed in the present study. Herrick et al. (2018) ruminally and abomasally infused butyrate and reported an increase in plasma BHB and a decrease in plasma glucose concentrations compared to a negative control. In the present study, we did not detect any effect on plasma BHB or glucose concentrations, making it more difficult to implicate butyrate supply as the likely cause of the increase milk de novo fatty acid yield. It may be that small increases in acetate, propionate, and butyrate supply combined to increase substrate available for milk fatty acid synthesis. Propionate that is absorbed into the blood is primarily converted to glucose through gluconeogenesis in the liver (Chan and Feedland, 1971), but a fraction of absorbed propionate is also used for fatty acid synthesis, and acetate is the primary substrate for fatty acid synthesis in the mammary gland of ruminants.

Free fatty acid concentrations typically increase during times of stress or when a cow is in a negative energy balance. Cows enrolled in this study were in peak lactation and were stressed due to multiple factors including mycotoxins (Quanz et al., 2021), but these factors do not explain why eCaG caused an increase in plasma FFA. Seymour et al. (2021) reported an increase in plasma FFA concentrations in cows supplemented with eCaG, but they also observed a tendency for increased concentrations of preformed fatty acids in milk. Considering both findings, they attributed this increase in FFA to the palm fat in the product (Seymour et al., 2021). We accounted for this in the present study by top-dressing the control group with an equivalent amount of palm fat; therefore, this component of the eCaG cannot explain the increased plasma FFA concentrations observed in our study. It is possible that with the increase in mammary de novo fatty acid synthesis there was decreased demand for FFA released into the blood by lipoprotein lipase. However, we are not aware of evidence that increased substrate

supply for fatty acid synthesis (e.g., acetate infusion) decreases uptake of preformed FA or increases plasma FFA concentrations (Urrutia and Harvatine, 2017; Urrutia et al., 2019). More research is needed to assess the physiological impacts of eCaG supplementation on lactating dairy cows, especially on fatty acid utilization and production.

We are not aware of published research investigating effects of calcium gluconate supplementation on health biomarkers in livestock. Health biomarkers were largely unaffected by treatment in this study. It was notable that temporal patterns for LBP, SAA, and haptoglobin (all acute phase proteins) differed markedly (Figure 3-3). Although often considered together, acute phase proteins clearly are regulated differently. It is worth noting that eCaG prevented a spike in LBP that was observed in week 9 in the Controls, perhaps related to the serious metabolic health challenges observed between weeks 4 and 9 (Table 3.3) and/or heat stress during that period of time (Quanz et al., 2021). Although not statistically significant, the number of cows removed for metabolic health problems was numerically greater for the Control vs. eCaG treatments, and it is important to consider that cows removed from the study were not represented in the blood data post-removal. Therefore, health biomarkers were potentially biased by differential removal rates across treatments, although mixed model analysis helps to account for these changes throughout the study. Health problems also caused us to deviate from the protocol, changing the basal diet midway through the study. This obstacle, in combination with the animals removed from study, may have impacted our findings and certainly decreased statistical power at the end of the study.

CONCLUSIONS

Supplementation of fat-embedded calcium gluconate tended to increase milk fat concentration with a tendency to reduce milk protein and lactose concentrations. Perhaps the

clearest impact of treatment observed in this study was the increase in secretion of de novo synthesized and mixed-source fatty acids in milk. Metabolic markers remained mainly unaffected except for an increase in plasma free FA in cows fed eCaG. No major differences were observed for health biomarkers even with the unexpected health challenges during the study. More work needs to be done to understand the impact of gluconate supplementation on fatty acid metabolism and milk fat synthesis.

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FIGURES

Table 0.1. Ingredient and chemical composition of total mixed ration (% of dry matter except where noted).

	Weeks -2–5	Weeks 6–12
Ingredients		
Corn silage A	16.7	6.29
Corn silage B	-	6.29
Alfalfa hay	20.8	20.9
Corn milling product[1]	17.4	17.5
Cottonseed	2.79	2.81
Corn grain	24.8	25.0
Expeller soybean meal[2]	12.2	12.2
Limestone	1.25	1.26
Sodium bicarbonate	0.85	0.86
Calcium salts of long-chain fatty acids[3]	0.83	0.84
Micronutrient pre-mix[4]	2.54	2.44
Wheat straw	-	3.51
Direct-fed microbial[5]	-	0.11
Nutrients		
DM, % as-fed	61.7	59.9
Net energy for lactation, Mcal/kg DM	1.69	1.63
Crude protein	18.5	19.4
Ether extract	5.03	5.01
Neutral detergent fiber	31.4	31.3
Acid detergent fiber	20.3	20.9
Ash	8.46	9.72
Ca	1.00	1.25
P	0.49	0.50

[1]Sweet Bran, Cargill, Blair, NE.

[2]Soy Plus, Landus Cooperative, Ames, IA.

[3]Essentiom, Arm & Hammer Animal Nutrition, Princeton, NJ.

[4]MegaLacR, vitamin E, salt, trace mineral, magnesium oxide, zinpro 4 plex c, zinpro 120, Rumensin 90, AjiPro L, Methionine MHA

[5]Biofix Plus Pro; Biomin America, Overland Park, KS.

Table 0.2. Cows removed from study

Cow ID	Treatment	Week of Study Removal	Parity	Reason for Removal
4821	Control	2	3	Lameness
6	Control	4	2	Metabolic
5148	Control	6	2	Metabolic
5138	Control	6	2	Metabolic
10	eCaG	8	2	Metabolic
4920	Control	9	3	Lameness
4985	eCaG	11	3	Mastitis

Table 0.3. Effects of encapsulated calcium gluconate (eCaG) on milk component production and efficiency of mid-lactation cows.

	Treatment			*P*-value		
	Control	eCaG	SEM	Treatment	Week	Treatment*Week
DMI, kg/d	29.9	30.1	0.44	0.75	<0.001	0.12
Milk yield, kg/d	47.2	48.3	0.72	0.13	<0.001	0.18
Fat, %	3.66	3.74	0.051	0.056	<0.001	0.94
Protein, %	2.91	2.87	0.020	0.08	<0.001	0.14
Lactose, %	4.72	4.69	0.014	0.07	<0.001	0.13
Protein yield, kg/d	1.36	1.39	0.021	0.38	<0.001	0.51
Fat yield, kg/d	1.75	1.80	0.037	0.19	<0.001	0.92
De novo fatty acids, g / 100 g FA	23.7	24.2	0.16	0.02	<0.001	0.29
Mixed fatty acids, g / 100 g FA	37.6	38.6	0.28	<0.01	<0.001	0.14
Preformed fatty acids, g / 100 g FA	38.7	37.3	0.38	<0.01	<0.001	0.19
De novo fatty acids, g/d	391	412	7.8	0.04	<0.001	0.80
Mixed fatty acids, g/d	625	654	13.9	0.05	<0.001	0.95
Preformed fatty acids, g/d	639	621	11.7	0.13	<0.001	0.50
Body condition score	3.01	3.03	0.033	0.62	<0.001	0.16
Body weight, kg	694	688	6.10	0.46	<0.001	0.48
ECM[1], kg/d	48.4	49.9	0.80	0.20	<0.001	0.54
Feed efficiency[2]	1.62	1.65	0.023	0.16	<0.001	0.35

[1]Calculated using the formula: = (0.327*milk yield) + (12.95*fat yield) + (7.65*protein yield).

[2]Calculated as ECM/DMI

Table 0.4. Effect of encapsulated calcium gluconate (eCaG) on plasma metabolite concentrations collected every other week for 12 weeks

	Treatment			*P*-value		
	Control	eCaG	SEM	Treatment	Week	Treatment*Week
Citrulline, µmol/L	138.9	141.4	2.15	0.42	<0.001	0.41
SAA[1], µg/mL	63.0	67.8	4.84	0.41	0.006	0.94
BHBA, µmol/L	502	522	16.9	0.37	0.004	0.79
Haptoglobin, µg/mL	1000	1030	67	0.76	<0.001	0.69
D-lactate, mmol/L	49.2	48.8	1.50	0.81	<0.001	0.41
LBP, ng/mL	497	439	48.3	0.37	<0.001	0.09
DAO, U/L	244	240	2.79	0.26	<0.001	0.16
NEFA, µmol/L	89.3	98.4	2.98	0.04	<0.001	0.33
Glucose, mg/dL	42.6	42.2	1.35	0.83	<0.001	0.15
Insulin, µg/mL	0.51	0.54	0.045	0.48	<0.001	0.30

[1]Log-transformed for analysis to achieve normal distribution of residuals; results are back-transformed.

Figures

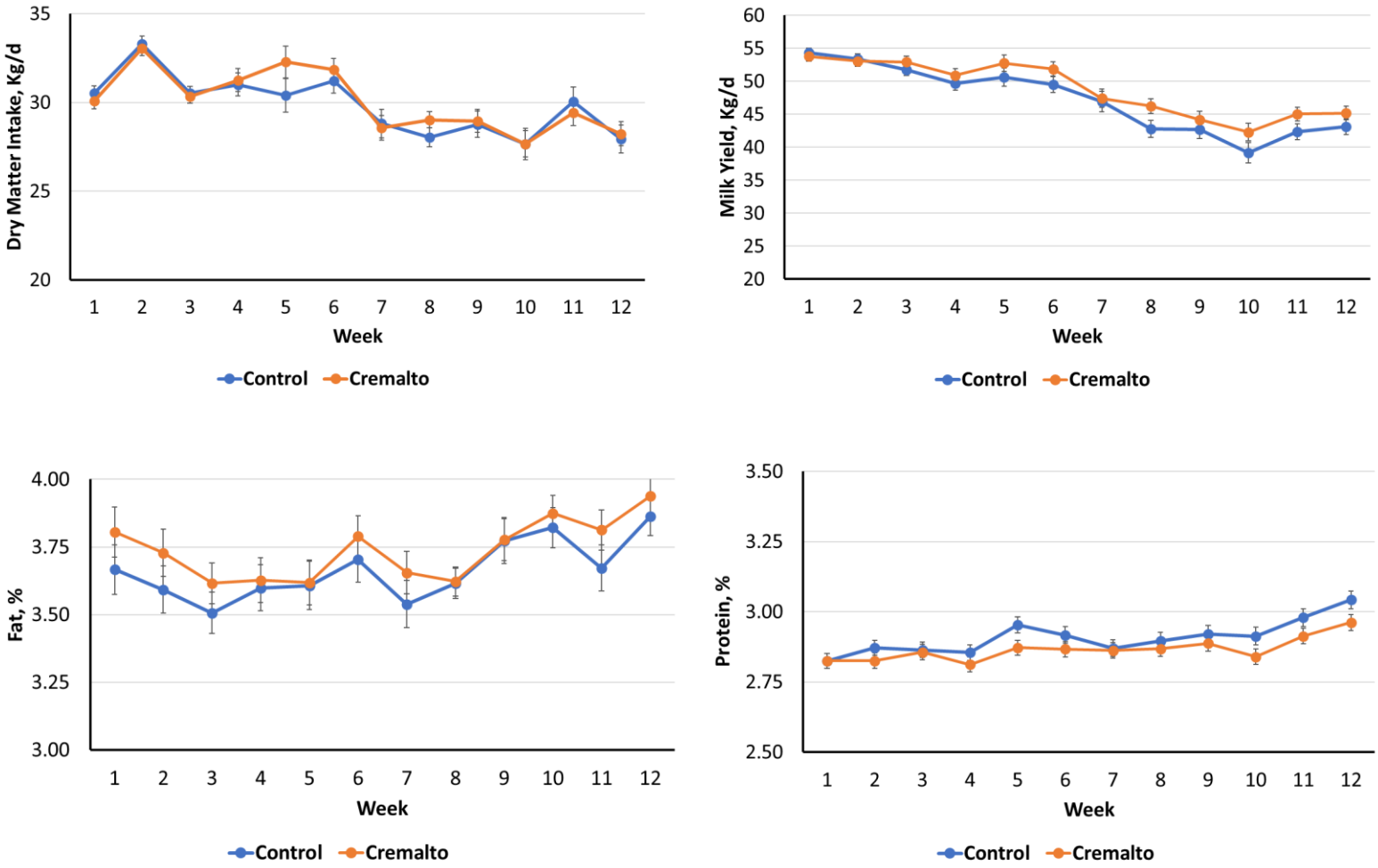


Figure 0-1. Effect of eCaG on A) DMI, B) milk yield, c) fat content, d) protein content

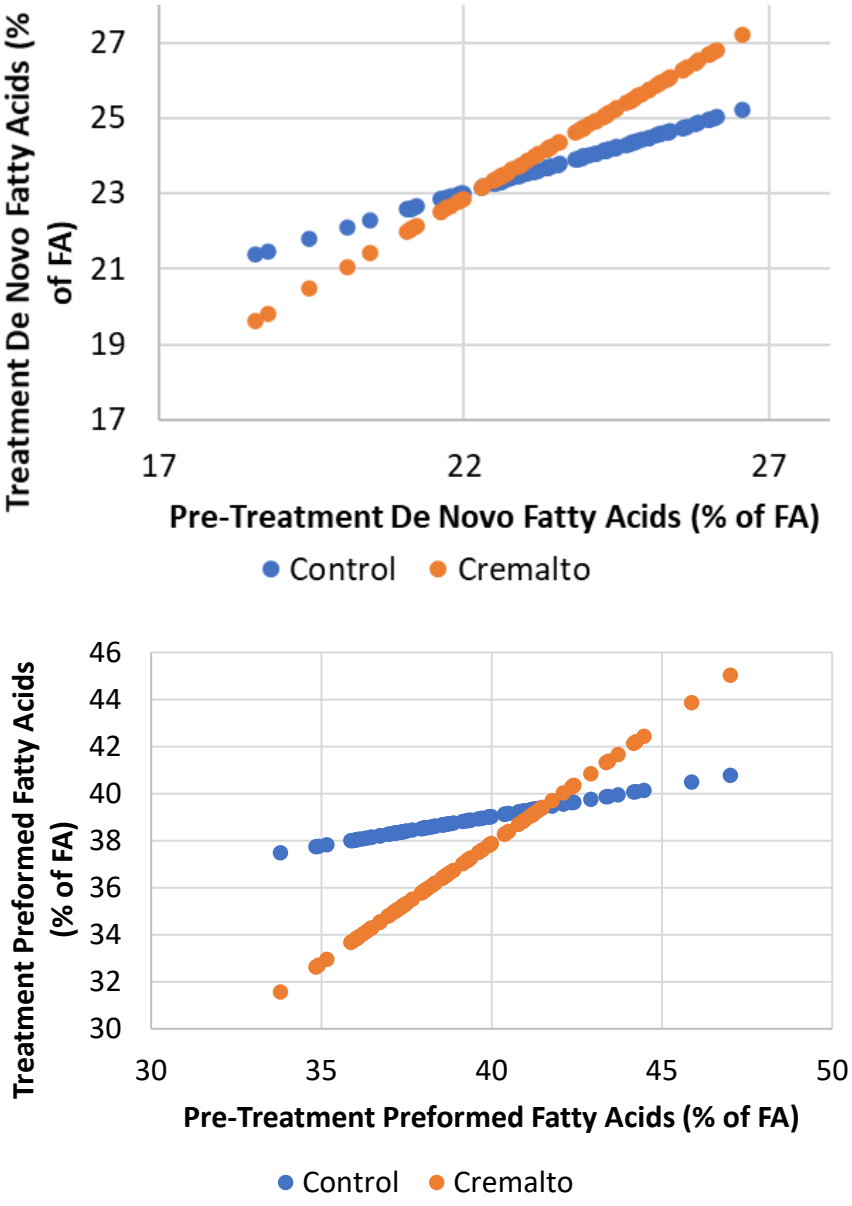
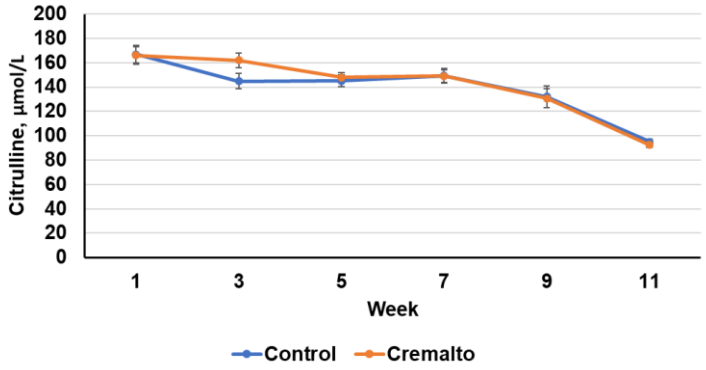
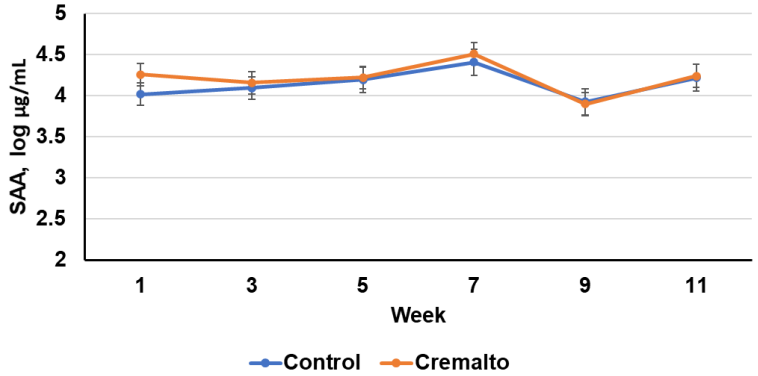
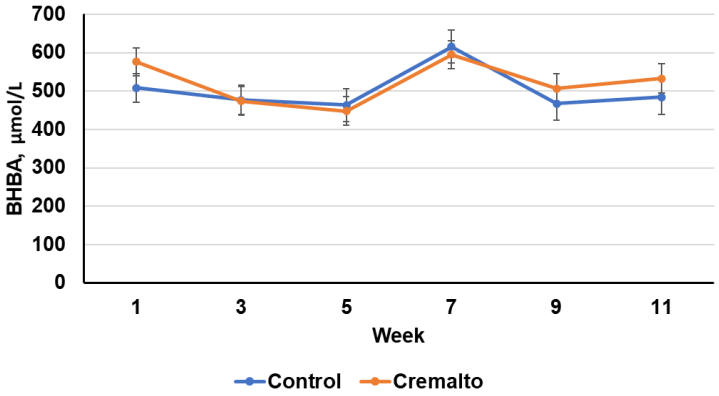
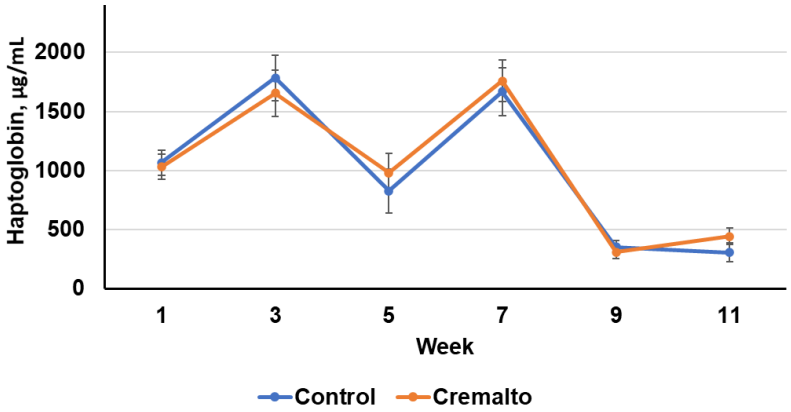


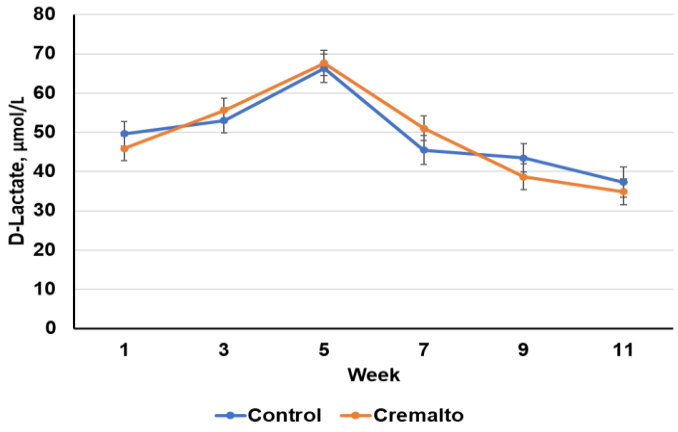
Figure 0-2. Covariate by treatment interactions for A) *de novo* fatty acids, B) preformed fatty acids, and C) DAO

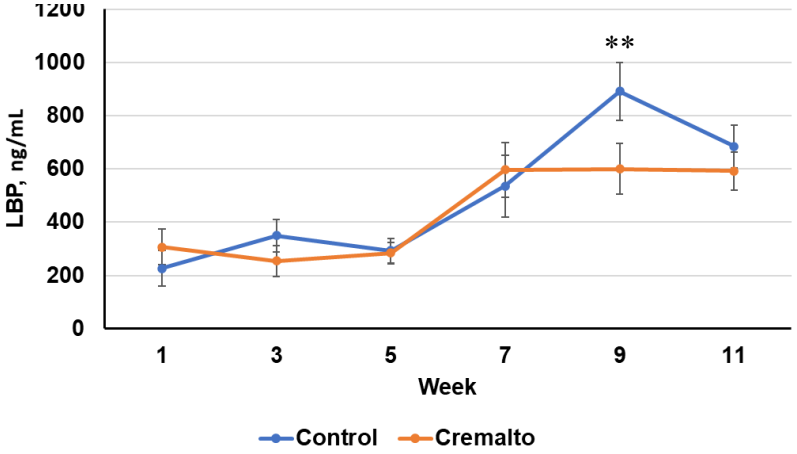

Citrulline, µmol/L
200
180
160
140
120
100
80
60
40
20
0
1
3
5
7
9
11
Week
Control
Cremalto

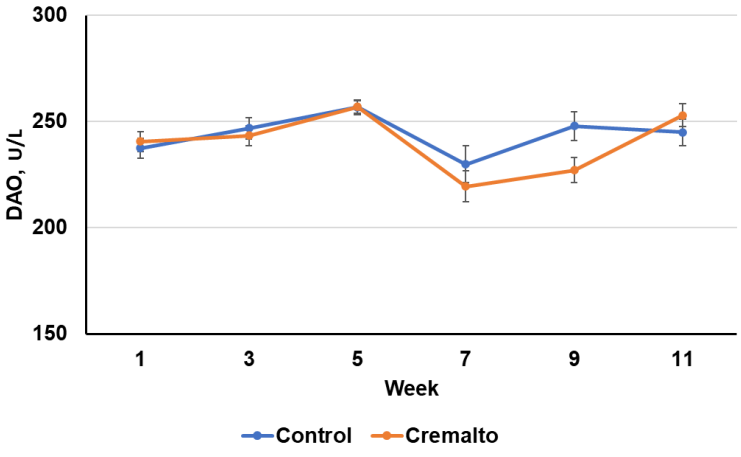

SAA, log µg/mL
5
4.5
4
3.5
3
2.5
2
1
3
5
7
9
11
Week
Control
Cremalto

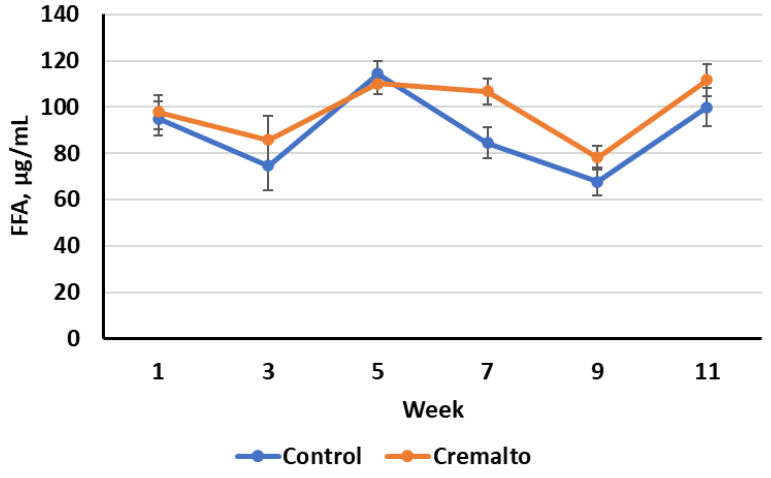

BHBA, µmol/L
700
600
500
400
300
200
100
0
1
3
5
7
9
11
Week
Control
Cremalto


Haptoglobin, µg/mL
2000
1500
1000
500
0
1
3
5
7
9
11
Week
Control
Cremalto


D-Lactate, µmol/L
80
70
60
50
40
30
20
10
0
1
3
5
7
9
11
Week
Control
Cremalto


LBP, ng/mL
1200
1000
800
600
400
200
0
**
1
3
5
7
9
11
Week
Control
Cremalto


DAO, U/L
300
250
200
150
1
3
5
7
9
11
Week
Control
Cremalto


FFA, µg/mL
140
120
100
80
60
40
20
0
1
3
5
7
9
11
Week
Control
Cremalto

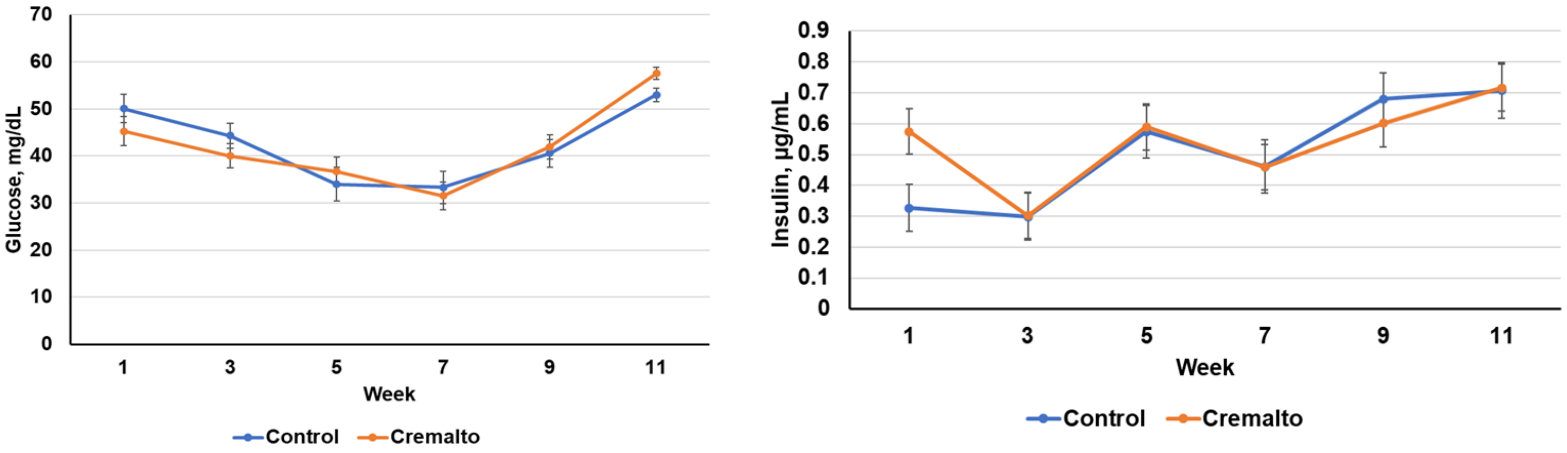


Figure 0-3. Treatment by time interactions of blood metabolites A) citrulline, B) serum amyloid A (SAA), C) BHB, D) haptoglobin, E) D-lactate, F) lipopolysaccharide binding protein (LBP), G) diamine oxidase (DAO), H) free fatty acids (FFA), I) glucose, and J) insulin. There was a tendency for a treatment × time interaction for LBP; ** $P < 0.05$ at designated time point**.**

Chapter 4 -Effect of *Lactobacillus* and *Bacillus* species on performance, health, ileal epithelium, and microbiota populations of the digestive tract in pre-ruminant calves through weaning

S. T. Quanz, K. A. Habib, K. J. S. Smith, A. J. Tarpoff, J. S. Thompson, C. S. Jones, S. E. Gragg, and B. J. Bradford.

INTRODUCTION

Throughout their life, livestock are exposed to a series of stressors. Calves undergo several management and physiological changes that introduce stress into their lives (Hulbert and Moisá, 2016). In modern practice, dairy calves are often transported shortly after birth to a calf grower. Here they may be mixed with calves from several different dairies, exposing them to unknown immunological challenges. Physiologically, calves slowly transition from a pre-ruminant to a ruminant digestive system as their rumen begins to develop and with the introduction of starter feed. Stressors such as shipping and co-mingling have been shown to physically change the microbiome and thin the epithelial lining of the digestive tract in monogastric animals. This thinning of the intestinal epithelium leaves the animal susceptible to opportunistic pathogens, such as Clostridia, pathogenic *E. coli*, and *Salmonella* (Peek et al., 2018).

The probiotic effects of *Bacillus* species in the intestinal tract of monogastric animals have been well documented. Various strains of *Bacillus* compete with pathogenic bacteria by competing for wall attachment (Jung et al., 2012), potentially upregulating the immune system (Huang et al., 2008), and producing antimicrobial compounds (Shobharani et al., 2015). In a similar fashion, lactobacilli can protect the intestinal epithelium against pathogenic bacteria by adhering to the intestinal epithelium (Lin et al., 2007). From a production standpoint, *Lactobacillus* supplementation in hogs has been shown to improve average daily gain (ADG) and feed efficiency (FE) compared to hogs fed antibiotics (Suo et al., 2012).

In ruminants specifically, *Lactobacillus* supplementation has been shown to decrease prevalence of pathogenic *E. coli* O157:H7 in feces of feedlot steers compared to a negative control (Brashears et al., 2003; Younts-Dahl et al., 2005; Peterson et al., 2007). However, in terms of animal performance, *Lactobacillus* supplementation has generally been unsuccessful in producing differences compared to a control (Brashears et al., 2003; Younts-Dahl et al., 2005; Peterson et al., 2007). For *Bacillus* species, supplementing feedlot cattle has been reported to increase dry matter intake (DMI), ADG, and body weight (BW) while decreasing the incidence of bovine respiratory disease (BRD; Smock et al., 2020). In dairy calves, *Bacillus* supplementation resulted in an increase in ADG compared to a control group (Sun et al., 2010).

It is not unreasonable to hypothesize that the combined probiotic effect provided by *Lactobacillus* and *Bacillus* would create a strong intestinal barrier against harmful bacteria that would subsequently result in improved performance. Therefore, the objective of this study was to assess the effect of *Lactobacillus* and *Bacillus* species on the performance, health, *E. coli* and pathogenic bacterial load, and the intestinal lining of pre-ruminant calves through weaning.

MATERIALS & METHODS

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

The study was conducted as a randomized complete block design with a 2 × 3 factorial arrangement. Sixty Angus × Holstein heifer calves (27.2 – 45.3 kg) were obtained from a commercial dairy in northeast Kansas. Calves were blocked by birth date and then randomly assigned to 1 of 2 treatments; 1) negative control (**Control**) milk or 2) calves received a combined 1.25×10^9 CFU per calf/d of *Lactobacillus* and *Bacillus* species (Certillus; Church & Dwight, Ewing, NJ) once a day in their milk (**Probiotic**). Calves were also randomly assigned within block to 1 of 3 harvest times (30, 60, or 90 d of age). Harvest dates were based on the

average birth date of each block, and each block was harvested within ± 2 d of their respective harvest date.

At birth, calves were tagged, weighed, had their navels dipped with iodine and hip height measured, and were housed in wooden hutches bedded with wheat straw. Calves were administered 1.9 L of colostrum replacer (Calf's Choice Total Gold, Saskatoon Colostrum Company Ltd., Saskatoon, SK, Canada) via esophageal tube within 2 h of birth. Within 12 h of birth, blood was collected by jugular venipuncture using a 20 ga needle into two 10-mL evacuated K_2EDTA-coated tubes and calves received 2.8 L of milk replacer containing their assigned treatment using a nipple bottle.

Forty-eight hours after birth, 3 mL of blood was collected via jugular venipuncture (BD Vacutainer SST Venous Blood collection tube) to measure serum Brix score using a refractometer (PA202 refractometer, Misco Products Corporation, Reading, PA). Calves that received a Brix score below 6.5 were removed from the study and replaced.

At 3 – 5 d of age, calves were transported 94 km to the Kansas State University Sheep and Meat Goat Unit (Manhattan, KS). Upon arrival, calves were randomly placed in individual hutches (Calf-Tel 24/74 Pro II/Deluxe II, Hampel Corp., Germantown, WI) with wheat straw for bedding. Within 48 hours of arrival, calves were given a physical examination by Kansas State University Veterinary Clinical Services to assess health and any abnormalities. Calves were intranasally vaccinated (Inforce 3, Zoetis, Parsippany-Troy Hills, NJ) for respiratory disease at 5 d of age.

Calves had ad libitum access to water. Calves were bottle fed 2.8 L of milk replacer (24% crude protein, 20% fat on a DM basis; Denkavit USA, Auburn, NY; Table 4.1) twice a day (0600 and 1800 h) mixed according to manufacturer's guidelines to contain approximately 14.5% (±

0.25) dry matter (DM) for the first 59 days of the study. On day 60, the remaining calves began a 10-d gradual weaning program where they were given 1.9 L of milk twice a day on d 60 – 64, 1.9 L once a day on d 65 – 69, and were completely weaned on d 70. Calves were provided with calf starter (20% crude protein, Countryside Feed LLC; Table 4.2) beginning on d 15 of the block. Calves were fed once in the morning with refusals measured 24 h later. Feeding rates were managed to target 0.23 kg of refusals each day. Control calves were fed milk replacer only, while probiotic calves received milk replacer supplemented with 1.25×10^9 CFU per calf/d of *Lactobacillus* and *Bacillus* species (Certillus; Church & Dwight, Ewing, NJ) once a day at the 0600 h feeding.

Calves were assessed daily for health. Calf eyes, ears, nose, and feces were scored from 0 – 3 by trained observers daily using the University of Wisconsin-Madison School of Veterinary medicine calf health scoring chart (Love et al., 2014). Sick animals were diagnosed by a veterinarian from the Kansas State University College of Veterinary Medicine who prescribed treatment as necessary.

Blood was collected on d 30, 60, 70, 80, and 90 through jugular venipuncture into two 10-mL evacuated K_2EDTA-coated tubes. Blood was centrifuged at 3,000 RPM for 10 min. Approximately 5 mL of plasma was collected into microcentrifuge tubes and frozen at -20°C for later analyses. Plasma was analyzed for the following gastrointestinal integrity and inflammatory biomarkers: citrulline (Homocitrulline/Citrulline ELISA Assay Kit, No. ab242292; Abcam, Cambridge, MA), diamine oxidase (Fluorometric Assay Kit, No. K496; Biovision Inc., Milpitas, CA), D-lactate (colorimetric Assay Kit, No. K667; Biovision Inc., Milpitas, CA), and the acute phase proteins haptoglobin (HP ELISA, Kit No. KA1850; Abnova, Taipei, Taiwan), serum amyloid A (ELISA Assay kit, No. TP-802; Tridelta Development Ltd., Greystones Co.,

Wicklow, Ireland), and lipopolysaccharide binding protein (Human LBP ELISA, Kit No. CKH113; Cell Sciences, Canton, MA).

Hip height and body weight were collected on d 28, 29, 30, 58, 59, 60, 68, 69, 70, 78, 79, 80, 88, 89, and 90. Weight and hip heights collected for 3 consecutive days were averaged and used for the 30, 60, 70, 80, and 90 data points. Hip height was assessed by measuring the distance from the ground to the top of the calf's hip bone with a retractable measuring tape.

Fecal samples were collected from the anus of each calf using a gloved hand and digital palpation within 12 h of birth (day 0; prior to treatment administration), and on days 30, 60, and 90 until their assigned harvest date. Fecal samples were placed in sterile Whirl-Pak® bags (Madison, WI) and held on ice (Day 30, 60, and 90 samples, 4 h) or under refrigerated conditions (Day 0 samples, 24 h). Samples were shipped overnight on ice to Agro Biosciences (Waukesha, WI) for microbial analysis.

As mentioned above, calves were randomly assigned to 3 different harvest days (30, 60, or 90 days of age) within treatment. Calves were euthanized using a captive bolt pistol (CASH Special, Model 4100R, Accles & Shelvoke, Sutton Coldfield, UK) followed by exsanguination of the jugular vein. Once confirmed deceased by a veterinarian, a standard bovine necropsy (Saunders, 2009) was performed to collect sections of digestive tract tissue and fluid. Tissue samples were collected from the colon, cecum, distal jejunum, proximal jejunum, ileum, duodenum, and rumen. Tissue sections were tied off with cotton string on either end to ensure digesta was not lost in the packaging and shipping process. Samples were placed into sterile Whirl-Pak® bags (Madison, WI), stored on ice and then shipped overnight for microbial analysis within 4 h of collection (Agro Biosciences, Waukesha, WI). Colon samples were tied off at the colon-rectal juncture and halfway up (~7.5 cm) the descending colon. Approximately a 20 cm^2

piece of cecal tissue was collected followed by 15 mL of cecal fluid into a separate bag. Ileal samples (~30.5 cm) were taken at approximately the midpoint after finding the ileal-cecal junction. Distal jejunum samples were collected by measuring one animal length from the ileum and taking approximately 30.5 cm section. Proximal jejunal samples were collected in a similar fashion, but one animal length was measured from the duodenum. Duodenal samples were harvested approximately 5 cm after the pyloric sphincter, collecting a ~30.5 cm sample. Rumen tissue approximately 20 cm^2 in size was collected from the ventral sac to ensure rumen tissue was submerged in rumen fluid. Finally, approximately 15mL of rumen fluid were squeezed from the mat layer of the rumen digesta. Approximately 2.54 cm of ileum tissue was cut from the digestive tract to be used for histology.

Digesta and fecal samples were shipped to Agro Biosciences (Waukesha, WI) for microbial analysis. The lab was blinded to treatments during the analysis process. Samples were enriched and enumerated using methods similar to Lucey et al. (2021). In brief, tissue samples were rinsed with 50 mL of sterile peptone (BD Difco, BD 218071, New Jersey). Five grams of sample were incubated for 37˚C for 24 hours and struck onto XLT4 agar (Remel, R459802, Kansas) for the identification of presumptive *Salmonella*. For *E. coli* and Clostridia counts, samples were enumerated on CHROMagarTM ECC (CHROMagar, EF322, New Jersey) and tryptose sulfite cycloserine (TSC) agar (Oxoid, CM0587, Hampshire) with D-cycloserine (400 mg/L), respectively. Up to 5 bacterial isolates were selected from the media for DNA analysis. DNA was purified with the Maxwell® HT Viral TNA Kit (Promega, AX2340, Wisconsin) according to the manufacturer's methods. Clostridia isolates were screened for the alpha toxin gene specific to *C. perfringens* (Yoo et al., 1997). *E. coli* isolates were tested for nine different virulence genes associated with pathogenic *E. coli*. The *E. coli* target genes were detected using

two multiplex PCRs, the first screening for *stx1*, *stx2*, *eae* and *ehxA* (Bai et al., 2012), a method optimized using four gene targets the volume of removed primers was substituted with water, and the second screening for *iroN*, *ompT*, *hlyF*, *iss* and *iutA* (Johnson et al., 2008). *C. perfringens* and pathogenic *E. coli* were estimated by multiplying the percentage of isolates tested positive for virulence genes against the entire population. Proximal jejunum tissue bacteria were also enumerated on De Man, Rogosa and Sharpe (MRS) agar (BD 288210, Thermo Fisher Scientific, Waltham, MA) for the quantification of lactic acid bacteria (LAB).

Ileal samples collected for histology were gently rinsed in 10% neutral buffered formalin (NBF), and then placed in a 10% NBF solution. After a minimum of 48 h, tissues samples were trimmed and 3 sections of samples were placed in tan cassettes (Leica Biosystems, Wetzler, Germany) and sent to Kansas State University Histology (Manhattan, KS) to be paraffin blocked. Once blocked, samples were shipped to Michigan State university Histopathology department (East Lansing, MI) for hematoxylin and eosin (H&E) staining. Samples were then analyzed for villus height and crypt depth using a Inverted microscope with 4 × magnification (Fisherbrand, Fisher Scientific Co., Hampton, NH). Images were taken with Fisherbrand camera (Fisher Scientific Co., Hampton, NH) digital imaging software (SeaView, Dexter Michigan).

STATISTICAL ANALYSIS

A calf from the Probiotic d 90 treatment, block 10, developed hindleg paralysis on d 32 of the study. The calf was euthanized by Kansas State University Veterinary Services for animal welfare reasons on d 35 and was then sent to the Kansas State University Veterinary Diagnostic Laboratory for necropsy. Necropsy results reported an abscess incasing the spinal cord. Spinal cord had become necrotic causing the paralysis. Samples and data from calf were kept in the dataset until d 30.

Data were analyzed using SAS 9.4 (SAS Institute Inc. Cary, NC) using the GLIMMIX procedure of SAS. Univariate procedure of SAS with conditional studentized residuals were used to check for normality and to remove outliers (>4 or < -4). Treatment effects were declared at $P \leq 0.05$ and tendencies for treatment effects at $0.05 < P \leq 0.10$. All data are expressed as least square means and standard error of the means.

Performance and plasma data were modeled with the GLIMMIX procedure of SAS with covariate data, treatment, time, and treatment × time as fixed effects. Block was used as a random effect. In addition, covariate × treatment was originally included in the models, but was removed if $P > 0.10$. Spline exponential was used for spatial power covariate structure to account for unequal sampling times for performance and plasma data.

Health and histological data were modeled with the GLIMMIX procedure of SAS with treatment, time, and treatment × time as fixed effects with block used as a random effect. Health data were analyzed according to the probability of a calf receiving a health score greater than 0. Fecal scores were also analyzed for greater than 0 and 1 since there was enough variation in the scores to do so. Health and histological data were modeled with autoregressive [AR(1)] or heterogenous autoregressive [ARH(1)] covariance structures, selected based on the least Bayesian Information Criterion value.

Intestinal microbial data were combined into 3 sections to increase power. Rumen fluid and rumen tissue samples were combined into the foregut (FG). Duodenal, proximal jejunum, distal jejunum, and ileum were combined into the midgut (MG). Cecal fluid, cecum tissue, and colon were combined into the hindgut (HG) section. Data were modelled with treatment, time, section, treatment × time, treatment × section, and time × section as fixed effects. A three-way interaction of treatment × time × section was originally included in the model but was removed if

$P > 0.10$. Block was included as a random effect. Data were modeled using compound-symmetry structure (CS) due to lower number of samples above the level of detection. Samples below the limit of detection (LOD) were not included in the model. Lactic acid bacteria (LAB) were enumerated from the proximal jejunum only. Due to the small sample size, the average and standard deviation for each treatment were calculated using Microsoft Excel.

Fecal microbial data were modelled with covariate data, treatment, time, and treatment × time as fixed effects. Block was included as a random effect. In addition, covariate × treatment was originally included in the models, but was removed if $P > 0.10$. Due to the low detection of Clostridia and *E. coli* on d 0 samples, data were analyzed with and without d 0 date to improve power analysis. Data were modeled using compound-symmetry structure (CS) due to lower number of data points. Samples below the limit of detection (LOD) were not included in the model.

RESULTS

Performance and efficiency results are shown in Table 4.3. There was no treatment effect for body weight, hip height, ADG, milk DMI, starter DMI, total DMI, or FE ($P > 0.05$), but time was significant for all variables ($P < 0.0001$). There was a tendency ($P = 0.08$) for a covariate × treatment effect for weight (Figure 4-2), pointing to negative effects of the probiotic on growth of calves with the greatest birth weights. There was a treatment × time interaction for starter DMI ($P = 0.003$) and total DMI ($P = 0.0004$), with probiotic supplemented calves demonstrating greater DMI between d 0 – 30 and control calves having greater DMI for the remainder of the study.

Calf health scores are shown in Table 4.4. There was no treatment, time, or treatment × time effect for eye (scores above 0), nose (scores above 0), or fecal (scores above 0 and 1) score ($P > 0.05$). The prevalence of ear scores above 0 tended ($P = 0.08$) to be greater for probiotic

treated calves (1.1%) compared to control treatment (0.5%). There was no time or treatment × time interaction for ear ($P > 0.05$).

Plasma data are shown in Table 4.6 and Figure 4.1. There was no treatment ($P > 0.05$) or treatment × time ($P > 0.05$) interaction for Haptoglobin, LBP, D-Lactate, SAA, Citrulline, or DAO. There was a time effect ($P < 0.05$) for D-lactate, SAA, Citrulline, and DAO. In general, D-lactate decreased from d 30 to 60 and then increased to d 90, SAA decreased steadily from d 30 to 90, Citrulline decreased from d 60 to 80 and then increased until d 90, and DAO increased from d 30 to 60 and then decreased to d 90. There was no time effect for haptoglobin or LBP ($P > 0.05$).

The main effects of digesta microbial data can be viewed in Table 4.7. There was no effect of treatment for *Clostridium* species, *Clostridium perfringens*, *Escherichia coli*, or pathogenic *Escherichia coli* ($P > 0.05$). There was a section ($P < 0.05$) and time ($P < 0.05$) effect for each variable measured. Section × time was not significant ($P > 0.05$) for *Clostridium perfringens*, *Escherichia coli*, and pathogenic *Escherichia coli*, but was for *Clostridium* species ($P = 0.049$). Statistical analyses were not run for *Salmonella* prevalence and concentration of LAB in the proximal jejunum due to low values. Prevalence of *Salmonella* was minimal for both control (2.2 ± S.D. 14.7%) and probiotic (1.1 ± S.D. 10.7%) treatments. In the proximal jejunum, control calves (5.6 ± 0.80 log cfu/g) had numerically higher concentrations of LAB than probiotic supplemented calves (5.4 ± S.D. 0.61 log cfu/g)

Fecal microbial data is shown in Table 4.8. For data analyses with d 0 covariate data, there was no effect of treatment for *Clostridium* species, *Clostridium perfringens*, *Escherichia coli*, or pathogenic *Escherichia coli* ($P > 0.05$). A time effect was observed for *Clostridium* species, *Escherichia coli*, and pathogenic *Escherichia coli* ($P < 0.05$), but not for *Clostridium*

perfringens ($P > 0.05$). In addition, there was a tendency for pathogenic *Escherichia coli* to vary according to treatment × time ($P = 0.0501$), but no interaction was observed for the other microorganisms ($P > 0.10$). For data analyses without d 0 covariate data there was a tendency ($P = 0.08$) for probiotic supplemented calves to have lower concentrations of *Clostridium* species while also having lower concentrations of *C. perfringens* than control calves. There were no treatment effects for *E. coli* or pathogenic *E. coli* among treatments. In addition, there was no treatment × time ($P > 0.10$) interaction for *Clostridium*, *C. perfringens*, and *E. coli*, but there was an interaction for pathogenic *E. coli* ($P < 0.05$).

Histological data is shown in Table 4.9. There was no observed effect of treatment on villus height or crypt depth ($P > 0.05$), but there was a time effect for both variables ($P < 0.05$). In addition, there was a treatment × time interaction for villus height ($P = 0.05$), but not crypt depth ($P > 0.05$).

DISCUSSION

Recently published research has begun to look at a combined supplementation of *Lactobacillus* and *Bacillus* species to calves (Lucey et al., 2021), but data is still limited. Due to calves being enrolled onto study at birth, we were unable to block by enrollment weight. Despite randomization, control calves tended ($P = 0.053$; 40.5 ± 0.62 kg) to have greater birth weights than probiotic calves (38.8 ± 0.62 kg). To account for this unplanned variation, birth weight was used as a covariate for ADG, milk DMI, starter DMI, total DMI, and FE. We did not detect major differences between treatments for any variables. Post-weaning growth performance appeared to be the most impacted by treatment, with treatment × time interactions for starter DMI and total DMI highlighting a slower rate of increase in starter intake after weaning for calves fed the probiotic. There was a time effect for all performance variables. This can largely be attributed the natural growth and/or management of calves that takes place over time.

We determined that treatment did not affect nose, eye, or fecal scores ($P > 0.05$). There was a tendency ($P = 0.08$) for probiotic supplemented calves to have a greater incidence of scoring above 0 for ear score. However, this is not considered to be a major indicator in the health of the calves because scores of one are still acceptable according to the scale that was used, and there were not enough ear scores above one to assess the variable at that level. There was enough data to assess fecal scores greater than one, with no differences found between treatments; however, a time effect was observed, with fecal scores decreasing from d 30 to 90. Lucey et al. (2021) observed diarrhea incidence in calves but reported no differences between control and probiotic supplemented calves. In feedlot cattle, BRD incidence was recorded for steers supplemented with *Lactobacillus* and *Bacillus* bacteria in combination with yeast ingredients compared to a negative control (Colombo et al., 2021). No differences were reported among treatments for BRD incidence; however, control cattle required a second dose of antimicrobials at a higher rate (Colombo et al., 2021). Illnesses of treated calves throughout the course of our study are included in Table 4.5. These data were not statistically analyzed due to the small study size and overall low incidence of illness in the calves; therefore, a statistical output would not have generated reliable, meaningful results. In general, study calves were relatively healthy which may have contributed to our lack of differences in other variables.

We observed no treatment effects for plasma health biomarkers ($P > 0.05$), but the effect of time was significant for DAO, SAA, D-lactate, and citrulline. Diamine oxidase in blood is thought to be the product of lysed enterocytes, and from epithelial cells in the intestines (Wollin et al., 1998). Our data demonstrated a sharp increase in DAO concentrations from d 30 to 60 and then a gradual decline from d 60 to 90. Recently, a number of studies have been published regarding decreased blood DAO concentrations and diarrhea incidence in calves (Fukuda et al.,

2019a; Fukuda et al., 2019b;Tsukano et al., 2020). Decreased DAO concentrations in humans have been linked to intestinal mucosal damage (Namiwaka et al., 2012; Miyosha et al., 2015). This may explain the low d 30 DAO concentrations, thereby indicating a time of gastrointestinal stress or a breakdown of intestinal health in calves. This also correlates to calf fecal score data (incidence > 1; Figure 4-3), which indicates that calves had higher fecal scores on d 30 and then gradually decreased to d 90. Serum Amyloid A decreased linearly from d 30 to d 90. Previous work involving calves reported similar decreases in SAA concentrations over time (Gånheim et al., 2007; Orro et al., 2008; Takemura et al., 2020). Published literature has stated that increases in calf SAA concentrations may be due to physical stressors (Alsmgeest et al., 1995; Orro et al., 2008). Since calves across treatments were housed in a similar manner for the duration of the study, it is unlikely that increases in SAA concentrations would be observed. In the present study, a decrease in D-lactate concentrations was observed from d 30 to 60, and then D-lactate increased continuously from d 60 to 90. D-lactate is mainly the product of microbial fermentation (Ewaschuk et al., 2004; Levitt and Levitt, 2020) and has been linked to calf diarrhea incidence (Lorenz, 2004; Ewaschuk et al., 2005; Wenge-Dangschat et al., 2020). The decrease in D-lactate concentrations from d 30 to 60 correlates to a decrease in fecal scores similar to DAO, but it does not account for the increase observed between d 60 to 90. Calf starter diets typically contain grains, such as corn, to increase diet energy, and the starter used for this study was no exception (Table 4.2). An increase in diet energy could potentially cause a shift in the site of digestion during and after weaning due to the increased starter intake. Grude (1999) concluded that there is a correlation between hindgut D-lactate production and blood D-lactate concentrations. Therefore, it is possible that more feed bypassed the rumen and was digested in the hindgut during and post weaning, thereby increasing D-lactate concentrations (Lorenz, 2009).

Citrulline is an organic compound involved in the urea cycle (Breuillard et al., 2015) that is synthesized in the small intestine and released by enterocytes (Sultana et al., 2003). It has been reported that calves with diarrhea have reduced concentrations of citrulline in their blood compared to healthy calves and citrulline could be used as an indicator of gut health (Gultekin et al., 2019). Given that lower concentrations of citrulline indicate gut dysfunction, the decrease in citrulline observed from d 60 to 80 suggests that calves were experiencing a period of intestinal breakdown. This is not altogether surprising given that this 20 d stretch consisted of the weaning phase and 10 d following it. This suggests that calves were stressed during the weaning phase, which has already been documented as a stressful period in a calf's life (Hulbert and Moisá, 2016).

Treatment, treatment × section, and treatment × time did not significantly impact intestinal microbial populations; however, each microorganism was associated with a time and section effect, and a time × treatment effect was observed for *Clostridium*. In general, microbial populations were different for each d (30, 60, and 90) of harvest. This is not surprising, as calves go through several physiological and nutritional changes during this time. Throughout the course of the study, the rumen began to develop and mature which would impact the microbial population of the digestive tract. In addition, on d 30, calves were consuming mostly milk, whereas on d 60 calves were consuming milk and starter feed, and on d 90 calves were only consuming starter feed. Therefore, it makes sense that microbial populations would differ, as a different substrate was being fed to the calves at each time point. When evaluating by section, microbial populations in the hindgut were consistently higher than the fore- and midguts for all microorganisms. The hindgut consists of the cecum and the colon which is the secondary site of digestion for ruminants. Previous research has reported that opportunistic bacteria, such as *E.*

coli and *Clostridium,* cannot compete in the fore- and midguts due to the competition for resources or the environment being unsuitable for growth (Cray Jr. and Moon, 1995; Grauke et al., 2002; Keen et al., 2010; Thorsteinsson et al., 2020).

Similar to the intestinal microorganisms, a treatment effect was not observed for fecal microorganisms. A time effect was observed for *Clostridium*, *E. coli*, and pathogenic *E. coli*, with a treatment × time interaction observed for pathogenic *E. coli*. In general, concentrations of bacteria decreased from d 30 to 90. This aligns with previous research that suggests that, as calf age increases, concentration of bacteria, specifically *E. coli*, decreases (Hoyle et al., 2004; Edrington et al., 2012). Runnels et al. (1980) postulated that animals develop a resistance to adhesion of these pathogenic bacteria as they grow older. Pathogenic *E. coli* fecal concentrations tended ($P = 0.097$) to be greater for probiotic supplemented calves on d 90 than control calves. There are several factors that could have influenced the fecal microbial outcomes. Firstly, covariate samples taken on d 0 had a small portion of that actually had microbe concentrations above the LOD. This limited covariant data limited the data points available for analysis which reduced power. The LOD for a majority of the study was 5,000 cfu/g which is approximately 3.7 log cfu/g, therefore any samples with concentrations below 3.7 log cfu/g did not produce a value. Having a LOD at 3.7 log cfu/g limited the number of positive samples detected. In addition, *Clostridium perfringens* and pathogenic *E. coli* values were extrapolated from the Clostridia and *E. coli* values, respectively. This was done by randomly selecting up to 5 cfu per plate and analyzing them for pathogenicity. Then the percentage of those cfus that were pathogenic were assumed to be representative of all the cfus counted.

We observed no treatment effect for villus height or crypt depth, but there was a time effect for both variables. The general trend for both variables were values increased as calves

were older. This aligns with trends from other variables that were measured throughout the course of this study. However, a previous study reported that ileum villus height were unaffected by calves between d 28 – 70 of age (Li et al., 2016). Bittrich et al., (2004) and Li et al. (2016) reported decreases in ileum villus height during the first month of a calf's life. Unfortunately, we were unable to collect a d 0 intestinal samples from the calves so we do not know how d 0 would have compared to the other harvest days.

CONCLUSIONS

This study provides preliminary evidence as to how this particular probiotic impacts calves through weaning. Minor treatment differences were observed with regards to performance and health. In addition, plasma, digesta and fecal populations, and ileal histology were observed to have no treatment effects. An additional study that is more appropriately designed to determine the effects on performance and health should be completed to more effectively evaluate the impact of this probiotic.

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FIGURES

Table 0.1. Milk replacer formulation and nutrient analysis (As-fed basis)1

Ingredient	% of Formulation
Whey protein concentrate	70.6
Refined animal fat & vegetable oil	18.2
Sweet whey powder	9.2
Vitamin and mineral mix	2.0
Nutrient Analysis, %	
Dry-matter, %	97.5
Crude protein	24.0
Crude fat	20.0
Crude fiber	0.0
Ash	6.5
NFE	47.0
Calcium	1.0
Phosphorus	0.7
Vitamin A, IU/lb	20000.0
Vitamin D, IU/lb	4000.0
Vitamin E, IU/lb	150.0

[1]Probiotic supplemented calves were given 1.25×10^9 cfu/calf/d of *Lactobacillus* and *Bacillus* species

Table 0.2. Calf starter formulation and nutrient analysis (As-fed basis)

Ingredient	% of formulation
35% protein pellet	43.0
Cracked corn	25.8
Whole oats	20.0
Molasses	5.0
Bovatec pellet	5.0
Golden Flo (flavored soybean oil)	1.2
Nutrient Analysis, %	
Dry-matter	90.0
Crude Protein	20.0
Undegradable intake protein	7.1
Degradable intake protein	12.7
Crude Fat	3.7
Crude Fiber	5.5
ADF	7.5
NDF	13.5
Ash	8.9
Calcium	1.4
Phosphorus	0.6
Lasalocid, g/ton	90.0

Table 0.3. Effect of probiotic on performance and efficiency of pre-weaned calves through weaning

	Treatment			*P* - Value		
	Control	Probiotic	SEM	Treatment	Time	Treatment × Time
Body weight, kg			1.05	0.10	<0.0001	0.67
30	52.6	52.7				
60	82.7	83.7				
70	96.3	95.8				
80	111.5	110.2				
90	125.3	121.7				
Hip Height, cm			0.42	0.26	<0.0001	0.82
30	83.0	82.9				
60	89.3	89.3				
70	92.1	93.2				
80	93.9	94.4				
90	96.1	96.9				
ADG, kg			0.04	0.22	<0.0001	0.19
0 -30	0.44	0.44				
31-60	1.00	1.02				
61-70	1.33	1.28				
71-80	1.51	1.44				
81-90	1.39	1.17				
Milk DMI, kg/day			0.003	0.59	<0.0001	0.74
0 -30	0.80	0.80				
31-60	0.81	0.81				
61-70	0.35	0.36				
71-80	-	-				
81-90	-	-				

Starter DMI, kg/day			0.07	0.76	<0.0001	0.003
15 -30	0.05	0.08				
31-60	0.76	0.70				
61-70	2.31	1.88				
71-80	3.92	3.30				
81-90	4.79	4.27				
Total DMI, kg/day			0.08	0.53	<0.0001	0.0004
0 -30	0.85	0.87				
31-60	1.58	1.48				
61-70	2.81	2.15				
71-80	3.93	3.29				
81-90	4.80	4.26				
FE, (ADG/DMI)			0.02	0.21	<0.0001	0.29
0 -30	0.53	0.51				
31-60	0.64	0.67				
61-70	0.48	0.56				
71-80	0.40	0.44				
81-90	0.29	0.26				

Table 0.4. Effect of probiotic treatment on calves receiving a health score > 0[1]

				P-Value		
	Control	Probiotic	SEM	Treatment	Time	Treatment × Time
Item, %						
Eye	3.0	3.7	1.0	0.44	0.80	0.48
Ear	0.5	1.1	0.3	0.08	0.22	0.61
Nose	2.5	3.1	0.4	0.32	0.15	0.78
Fecal	35.9	34.7	3.9	0.65	0.14	0.29
Fecal[2]	7.2	8.8	0.01	0.21	0.02	0.54

[1]Calves health scored on a scale of 0 – 3 using the University of Wisconsin calf health scoring sheet

[2]Score of calves receiving a fecal score >1

Table 0.5. Incidence of treatment between study treatment groups

	Control	Probiotic
Morbidity		
Diphtheria	0	1[1]
Pneumonia	0	1
Abomasitis	1	0
Abscessed naval	0	1[2]
Thickened naval	1	4
Lameness	1	2[3]
Coronavirus	1	0
Diarrhea	3	3
Total	**7**	**12**

[1]Calf required tracheostomy surgery due to paralyzed larynx
[2]Calf required umbilical surgery due to infected/abscessed naval
[3]One calf became lame due to spinal abscess and required removal from study and euthanasia

Table 0.6. Effect of probiotic on plasma inflammation biomarkers of pre-weaned calves through weaning

				P-Value		
	Control	Probiotic	SEM	Treatment	Time	Treatment × Time
Haptoglobin, μg/mL	5.37	4.96	0.56	0.60	0.91	0.55
LBP, μg/mL	1.20	1.10	0.20	0.50	0.64	0.82
D-Lactate, μM	15.40	14.40	1.69	0.56	<0.0001	0.97
SAA, μg/mL	29.47	43.22	7.69	0.20	0.0003	0.86
Citrulline, μM	434.84	426.15	15.59	0.69	<0.0001	0.22
DAO, mU/mL	6.56	6.44	0.41	0.82	<0.0001	0.56

Table 0.7. Effect of probiotic on intestinal microbial populations of pre-weaned calves through weaning

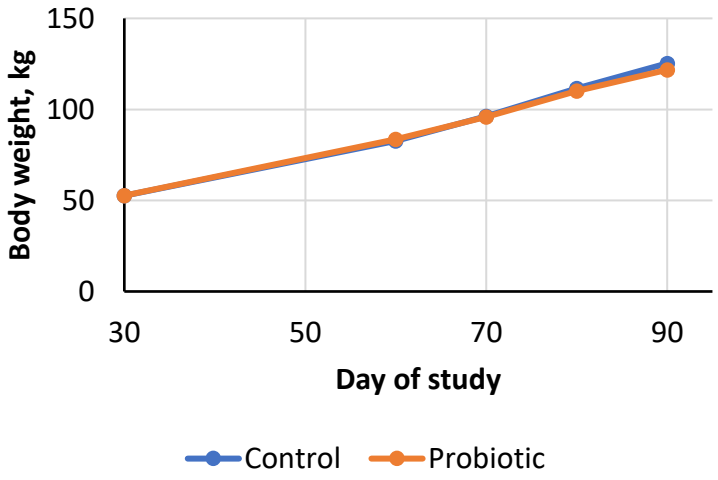
				P-Value			
	Control	Probiotic	SEM	Treatment	Time	Section	Time × Section
Clostridium, log cfu/g	2.31	2.13	0.14	0.34	0.0017	<0.0001	0.049
Clostridium perfringens, log cfu/g	2.16	1.96	0.14	0.32	0.0007	<0.0001	0.11
Escherichia coli, log cfu/g	4.00	4.12	0.16	0.58	<0.0001	<0.0001	0.17
Pathogenic *E. coli*, log cfu/g	3.53	3.77	0.22	0.38	0.0001	<0.0001	0.57

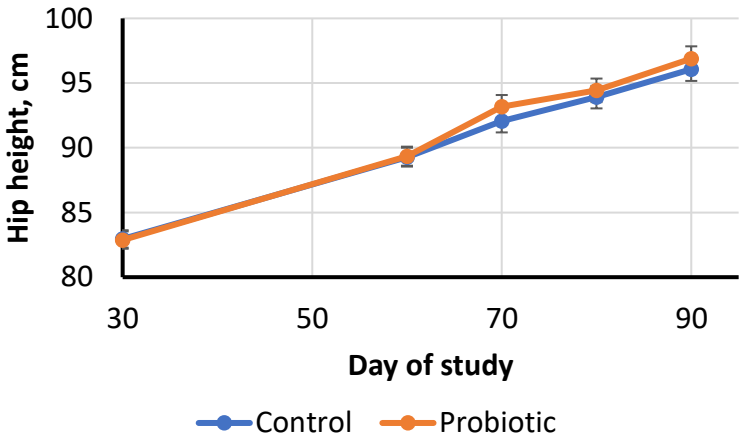
Table 0.8. Effect of probiotic on fecal microbial populations of pre-weaned calves through weaning

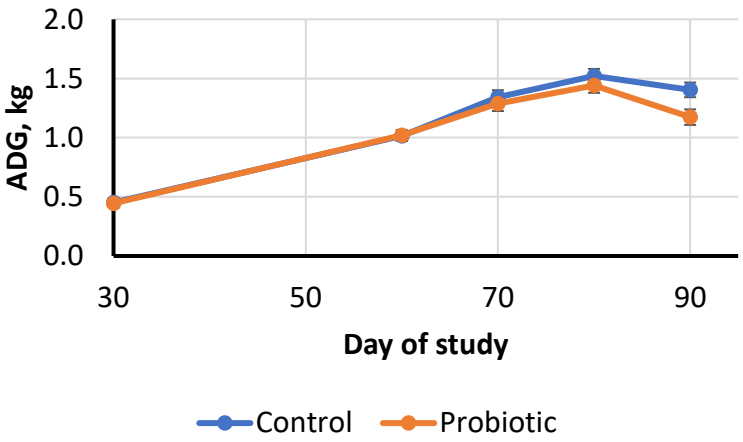
				P-Value		
	Control	Probiotic	SEM	Treatment	Time	Treatment × Time
With d 0 covariate						
Clostridium, log cfu/g	2.67	1.85	0.41	0.15	0.02	0.54
Clostridium perfringens, log cfu/g	2.73	1.52	0.32	0.17	0.38	0.59
Escherichia coli, log cfu/g	6.13	6.39	0.38	0.63	0.0009	0.96
Pathogenic *E. coli*, log cfu/g	5.46	6.60	0.69	0.33	0.04	0.0501
Without covariate						
Clostridium, log cfu/g	2.42	2.03	0.21	0.08	0.0001	0.93
Clostridium perfringens, log cfu/g	2.37	1.69	0.28	0.02	0.02	0.82
Escherichia coli, log cfu/g	6.01	6.26	0.21	0.25	0.0001	0.19
Pathogenic *E. coli*, log cfu/g	5.55	5.67	0.22	0.60	0.0001	0.04

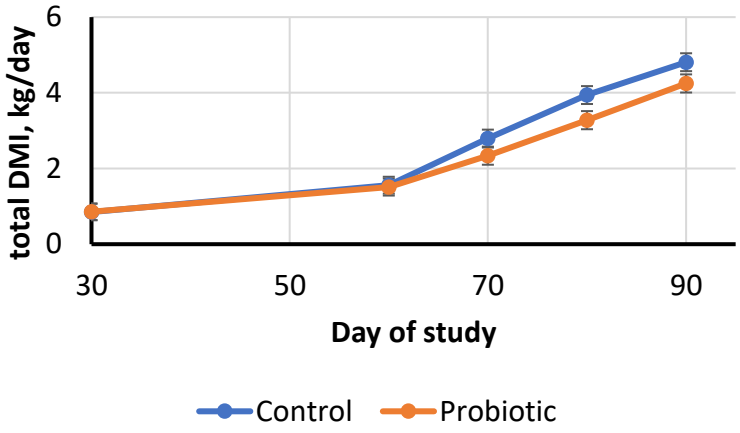
Table 0.9. Effect of probiotic on ileum villus height and crypt depth in pre-weaned calves through weaning

				P-Value		
	Control	Probiotic	SEM	Treatment	Time	Treatment × Time
Villus height, µm	3.70	3.77	0.18	0.70	0.0001	0.05
Crypt depth, µm	1.80	1.90	0.11	0.37	0.0007	0.95









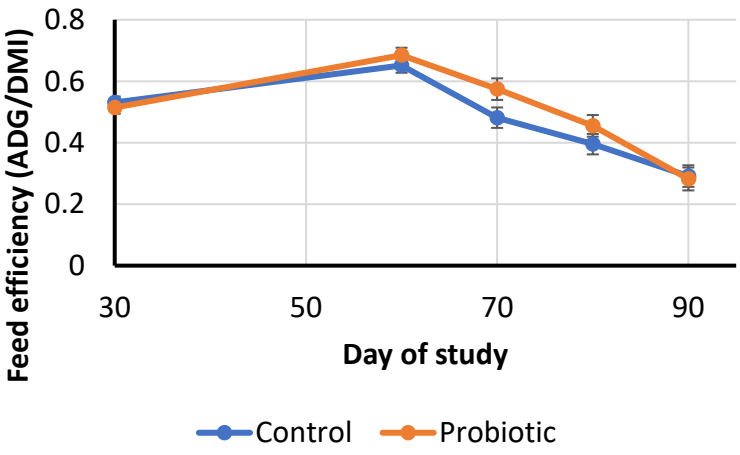


Figure 0-1 Effect of Probiotic supplemented to pre-weaned calves through weaning on: A) Body weight B) Hip Height C) ADG D) total DMI E) FE

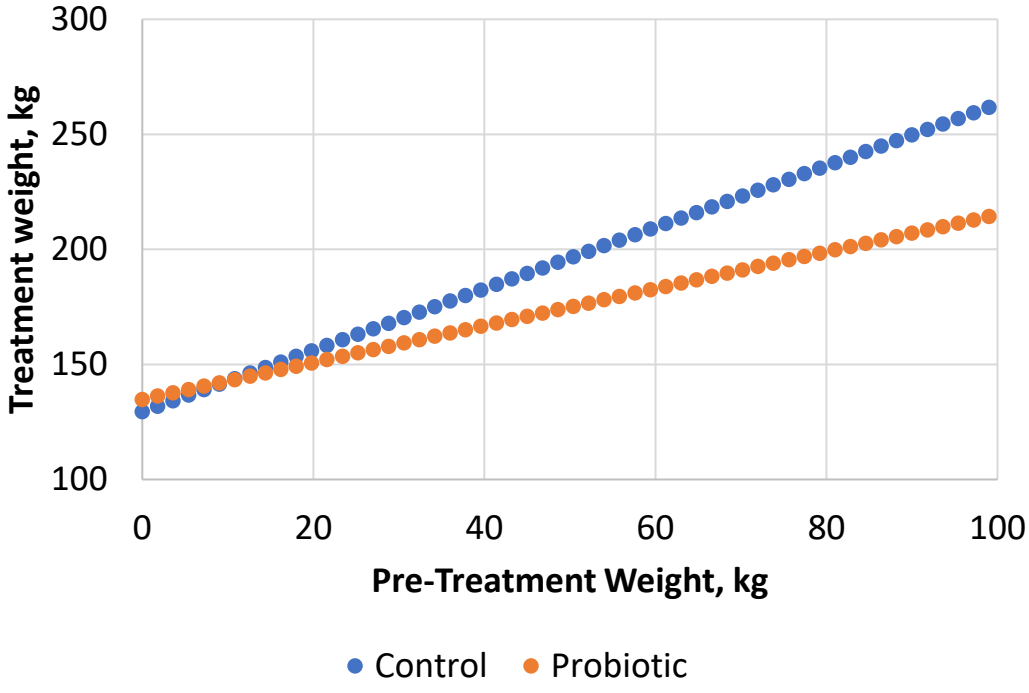


Figure 0-2. Covariate by treatment interaction for weight

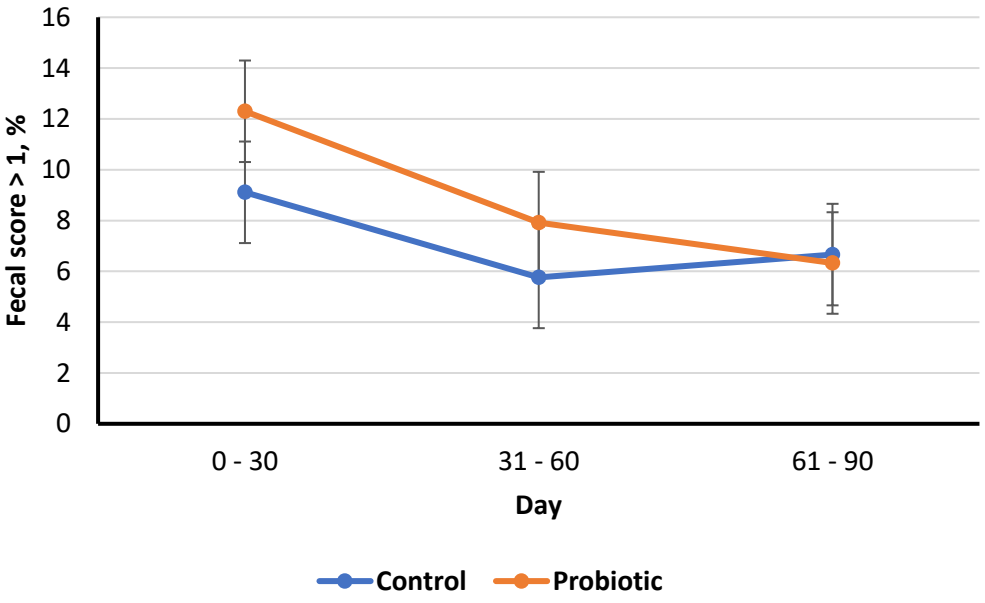


Figure 0-3. Effect of probiotic supplementation on pre-weaned calves fecal score incidence (>1) through weaning.

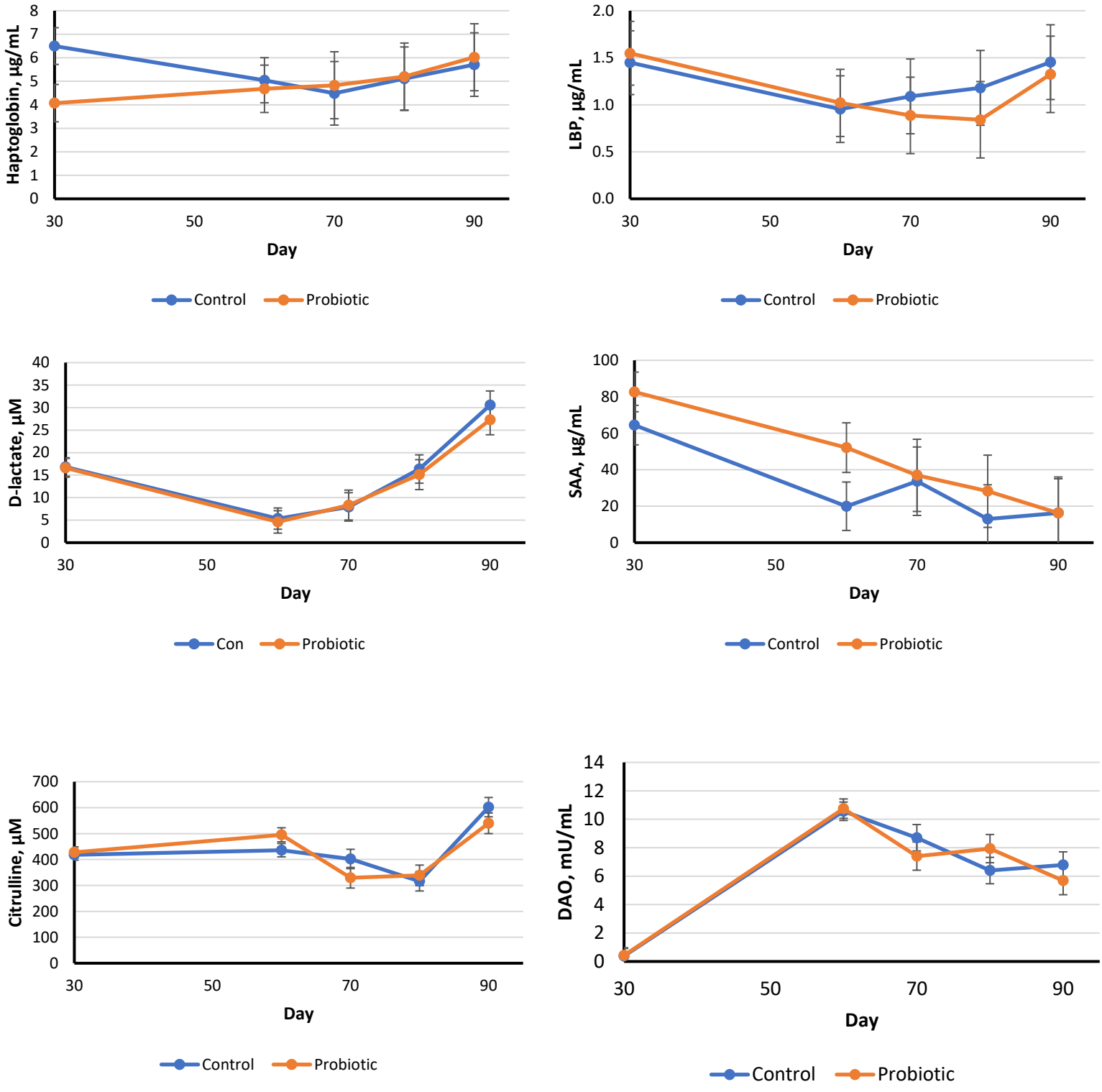
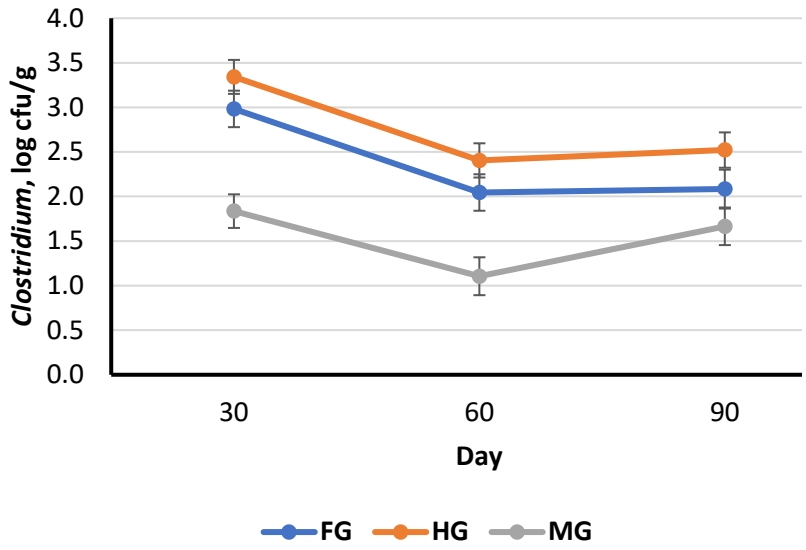
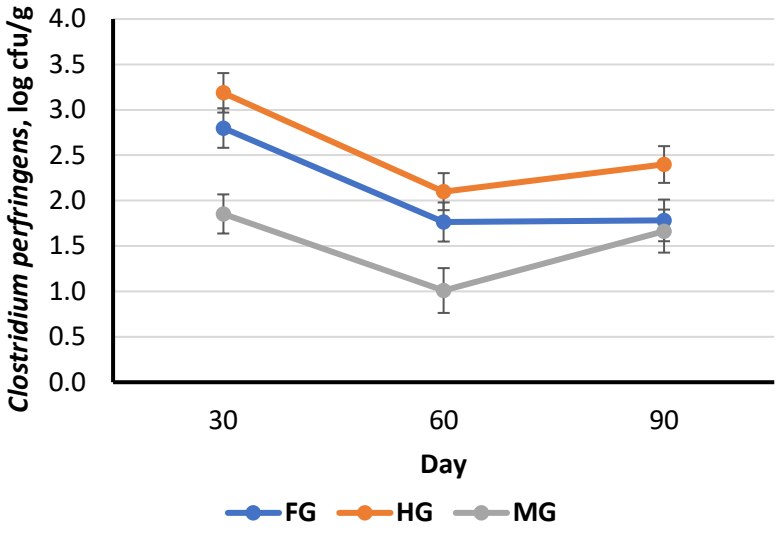
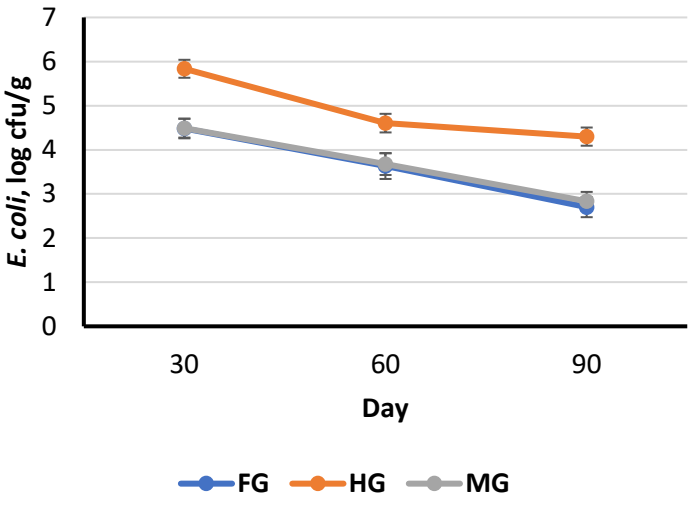


Figure 0-4. Effect of probiotic supplementation on pre-weaned calves through weaning on: A) Haptoglobin B) LBP C) D-Lactate D) SAA E) Citrulline F) DAO







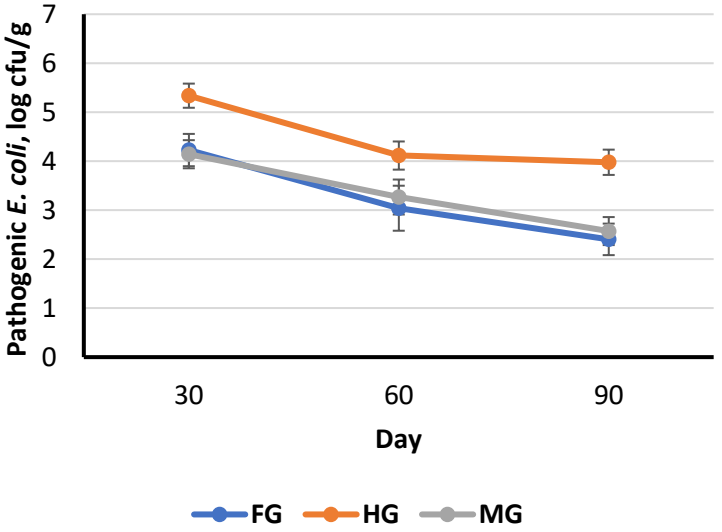


Figure 0-5. Effect of time of harvest and section of digestive tract on microbial populations: A) *Clostridium* species B) *Clostridium perfringens* C) *Escherichia coli* D) pathogenic *Escherichia coli*. Foregut =FG, Midgut = MG, Hindgut = HG.

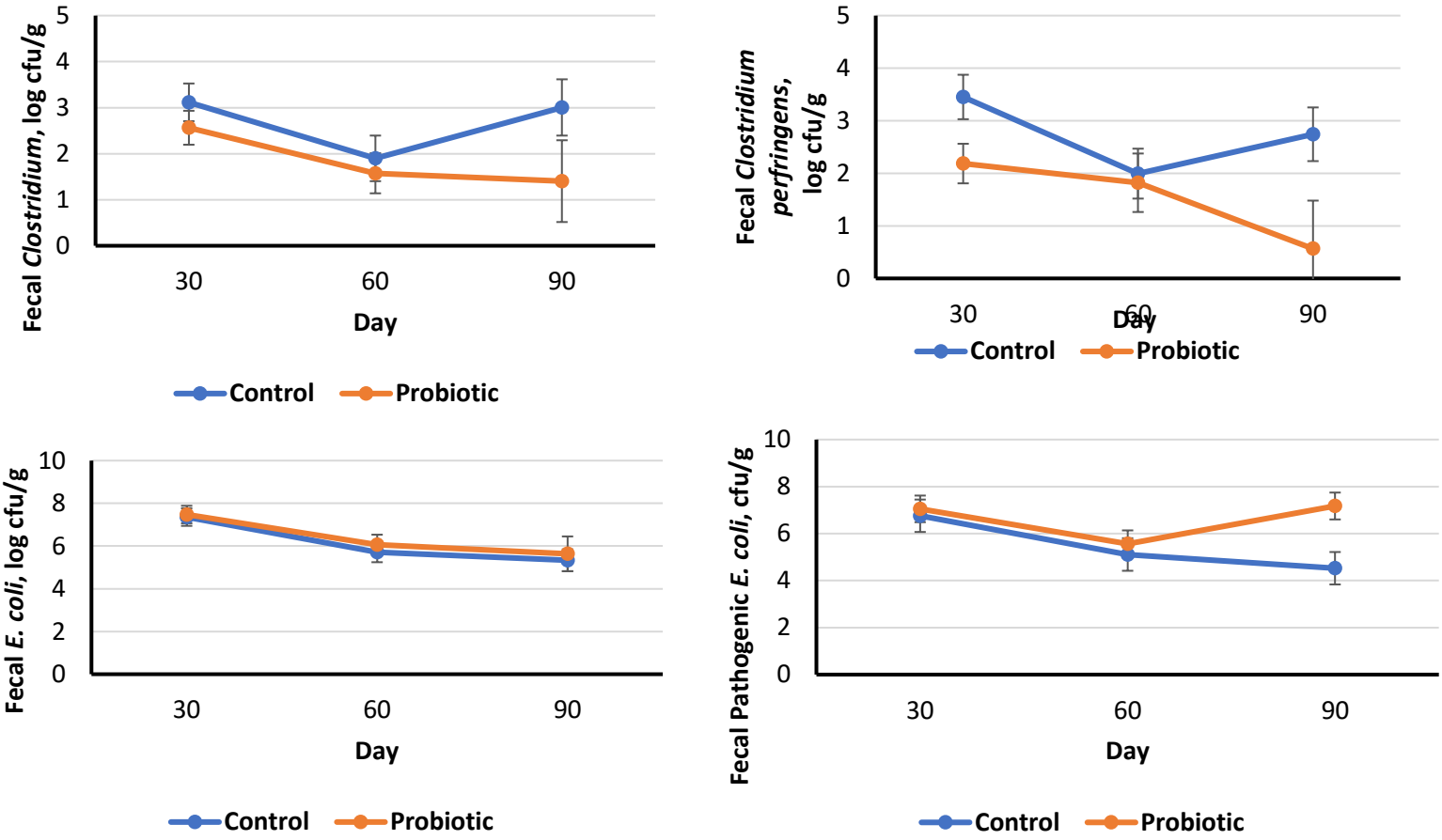


Figure 0-6. . Effect of treatment on fecal pathogenic bacteria of pre-weaned calves through weaning. A) *Clostridium* species B) *Clostridium perfringens* C) *Escherichia coli* D) pathogenic *Escherichia coli.*

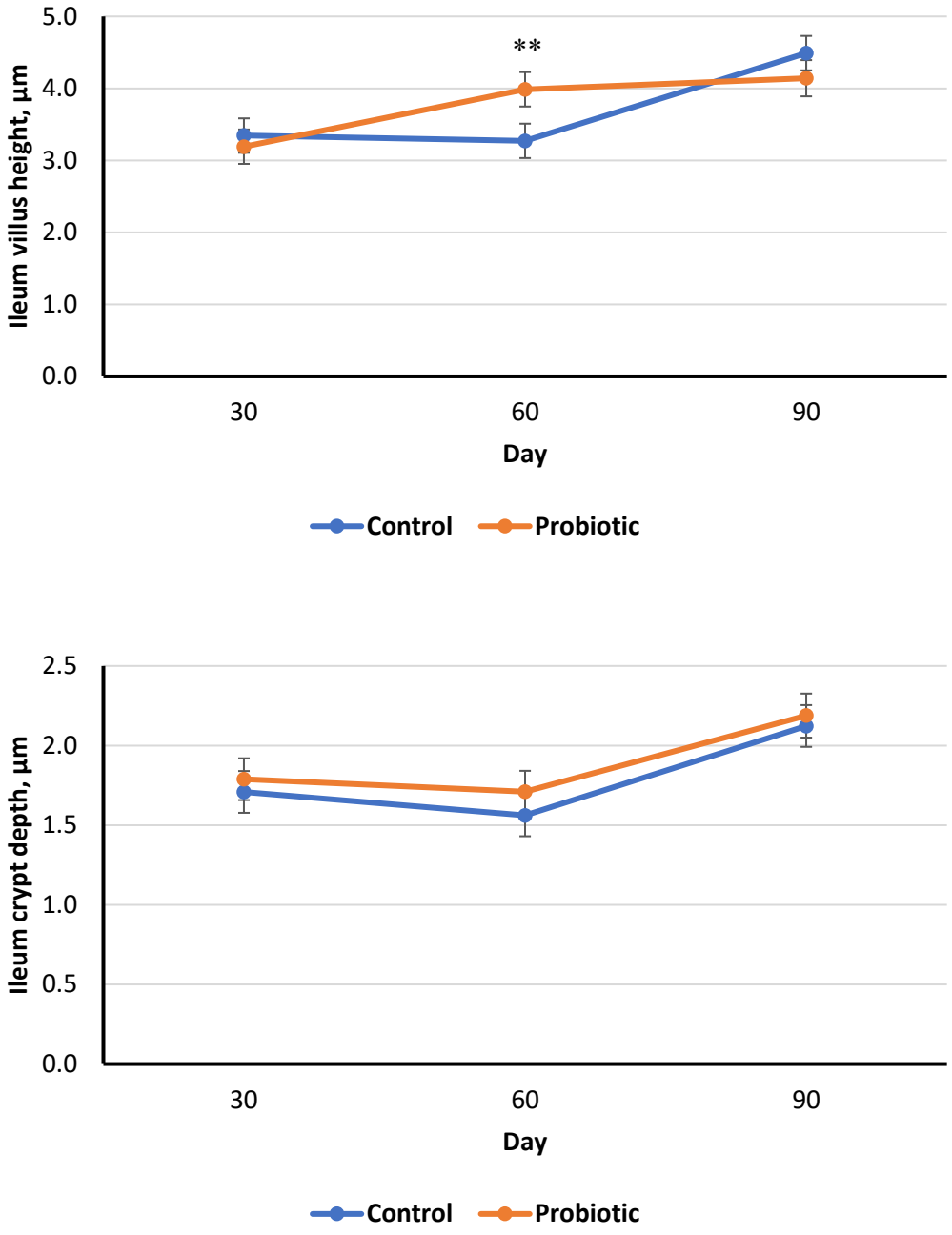


Figure 0-7. Effect of Probiotic supplementation on ileum histology of pre-weaned calves through weaning. A) Villus length, B) Crypt depth. ** Tendency (*P* = 0.097) for probiotic supplemented calves to have greater villus height on d 60.