Effects of *Macleaya cordata* extract on growth, health, gut morphology, and gut microbiota composition of calves

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This work is dedicated to my family, who inspired and supported me throughout my education. Without their love and sacrifice this would not have been possible.

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ABSTRACT

The use of antibiotics in animal agriculture is under significant scrutiny from the public as well as some scientific communities. This perception encourages identifying alternative feed additives with antimicrobial properties. Extracts from medicinal plants such as *Macleaya cordata*, commonly known as plum poppy, have been considered as a potential alternative. The implications of feeding *Macleaya cordata* extract (MCE) in cattle are poorly understood. Objectives of the present study were 1) to evaluate serum chemistry, hematology, and the tissue residues of calves fed graded levels of dietary MCE, and 2) to determine the effects of MCE on feed intake, weight gain, gut development, and gut microbiota composition of calves. Forty dairy × beef crossbred calves were assigned randomly to control (CTL), and low (LW), medium (MD), and high (HG) doses of a commercial *Macleaya cordata* extract preparation. The MCE was delivered mixed in milk replacer (MR) or top-dressed on starter during pre- (5 to 49 d of age) and post-weaning (50 to 95 d of age) periods, respectively. The corresponding doses in pre- and post-weaning periods were 0, 2, 5, 10, and 0, 4, 10, 20 g/calf/d, respectively. Feed intake was recorded daily. Body weight was recorded, and blood was collected at 5, 49, and 95 d of age. Calves were euthanized at 95 d of age to collect digesta, and tissues of the rumen and the small intestine. Additionally, we collected liver, kidney, skeletal muscle, and adipose tissue samples to analyze sanguinarine and chelerythrine residue concentrations. Those results are not presented in this thesis as the analyses are still underway. The blood was analyzed for clinical chemistry and hematology. A histology analysis was conducted to assess the morphology of rumen papillae and the intestinal epithelium. The DNA was extracted separately from rumen liquid (RL), rumen solids (RS), jejunal mucosa, and jejunal digesta, and subjected to 16S rRNA gene sequencing in an Illumina MiSeq platform. The effects of MCE dose on daily feed intake and the other response variables were analyzed using

the MIXED and GLM procedure of SAS, respectively. Regardless of the dose, calves fed MCE had greater MR intake compared to CTL pre-weaning ($P < 0.001$). Starter intake and thus dry matter intake (DMI) had a cubic relationship with MCE dose, where LW had lower starter intake and DMI than CTL, MD, and HG both pre- and post-weaning ($P < 0.050$). The HG tended to have greater DMI than CTL pre-weaning ($P = 0.066$). Body weight (BW) and average daily gain (ADG) were not affected by MCE dose both pre- and post-weaning. All blood chemistry and hematology parameters across treatments were within the reference ranges of healthy calves indicating no adverse effects of MCE. Blood creatinine concentration increased linearly with MCE dose within the healthy range indicating a potential to have increased muscle mass and thus high carcass dressing percentage as MCE dose increases. When HG dose was assessed against the CTL, MCE improved the morphology in both the rumen and the small intestine. The MCE supplement increased rumen papillae length ($P = 0.013$), and small intestine villus height ($P < 0.030$) and villus height to crypt depth ratio (VCR, $P < 0.070$). The HG did not affect volatile fatty acid profiles in the rumen. We did not observe HG affecting the richness and the diversity of rumen microbiota or microbiota communities in the small intestine. Nonetheless, the MCE supplement changed the relative abundance of several individual species in the rumen as well as the small intestine. The abundance of *Blautia caecimuris* decreased in RL ($P = 0.040$) and RS ($P = 0.010$) for HG and was negatively correlated with ADG. On the other hand, the abundance of *Gastranaerophilales_UC* which was positively correlated with DMI ($r = 0.55$, $P < 0.020$) and ADG ($r = 0.61$, $P < 0.010$) was lower in HG than CTL in jejunal digesta. In jejunal mucosa, nine species were affected by the MCE supplementation, but none of those species were correlated with DMI, ADG, villus height, or VCR. The MCE supplement, however, decreased the abundance of *Clostridioides difficile*, a zoonotic pathogen for which calves are considered as significant reservoirs. Considering together,

the doses of MCE tested in the present study are safe in calves and feeding MCE can be beneficial as it improves gut morphology and decreases the abundance of zoonotic bacteria in the feces of calves.

CHAPTER 1. GENERAL INTRODUCTION

There has been increased focus on improving antimicrobial stewardship in animal agriculture as a result of growing concerns about antimicrobial resistance (Gustafson and Bowen, 1997; Wemette et al., 2021). Recent studies and surveys point out that modern consumers are highly conscious about their purchasing decisions and prefer to avoid foods produced by utilizing pesticides and antibiotics (Bemporad et al., 2007). This causes producers to seek alternatives for such compounds that would be considered "natural" by consumers. *Macleaya cordata* commonly called plume poppy is a plant native to China and Japan, where it is used for medicinal purposes. *M. cordata* belongs to the tribe *Chelidonieae* members of this tribe, *M. cordata* produces colored latex rich in alkaloids (Pencikova et al., 2011). Among those, isoquinoline alkaloids including sanguinarine and chelerythrine possess antimicrobial and anti-inflammatory properties (Kudera et al., 2020). Previous studies using pigs demonstrated that dietary supplementations of *Macleaya cordata* extract (MCE) improved gut morphology characterized by longer villi and increased villus height to crypt depth ratio in the small intestine (Kantas et al., 2014; Chen et al., 2018). Studies with broiler chickens also showed increased villi height that was associated with increased ADG for MCE supplements (Liu et al., 2020). Those studies, however, showed no effect on feed intake indicating that MCE positively affected the growth by improving the nutrient utilization efficiency. Even though such effects of MCE are yet to be described for cattle, a dietary MCE supplementation increased the density (number of papillae per cm^2) and the cross-sectional area of rumen papillae in lambs (Lima et al., 2019). Because the possibilities for manipulating the gut morphology are limited primarily to the neonatal stage (Lyons et al., 2020), beginning to feed MCE immediately after they are born was hypothesized to improve permanently the rumen papillae and the intestinal morphology.

The microbiota community composition is another aspect of gut development that can respond to MCE. Zdunczyk et al. (2010) demonstrated a potential for seeing such responses as dietary MCE significantly altered volatile fatty acid (VFA) profiles in the cecum of broiler chickens. Liu et al. (2016) demonstrated later that MCE increased the abundance of beneficial bacteria including *Lactobacillus* and *Bifidobacterium* in the caecum, whereas it decreased the abundance of pathogenic bacteria including *E .coli* and *Salmonella* in the ileum of piglets. The impact of MCE on the gut microbiota composition of cattle is poorly understood. On the other hand, given the complexity of the gastrointestinal tract of ruminant animals, the findings of monogastric animals cannot be extrapolated directly to cattle. If MCE possesses similar effects on the gut microbiota communities in cattle, feeding MCE would enhance the sustainability of dairy and beef production by mitigating antibiotic use and fecal excretions of pathogenic. The antimicrobial properties MCE should not, however, adversely affect rumen microbiota. Therefore, new studies focused on dietary MCE supplements to cattle need to assess the impact of MCE on microbiota and their functions in the rumen. Moreover, plant extracts such as MCE contain a myriad of secondary plant metabolites that can cause toxicities in animals (Walterova et al., 1980) as well as humans consuming foods (e.g., meat and milk) produced with those animals. Therefore, a comprehensive safety assessment including serum chemistry and hematology analyses, and determination of MCE residues in edible in response to graded levels of dietary MCE is necessary. The objectives of the research project described in this thesis are 1) to evaluate serum chemistry, hematology, and the tissue residues of calves fed graded levels of dietary MCE, 2.) to determine the effects of those MCE doses on feed intake, ADG, and feed efficiency, 3.) to determine the effect of dietary MCE supplementation on rumen VFA profiles, and the epithelial morphology and microbiota community composition in the rumen and the small intestine of calves.

THESIS ORGANIZATION

This manuscript will discuss the effects of a *Macleaya cordata* extract preparation on performance, health and safety, gut morphology and gut microbiome composition of calves. The following chapter, CHAPTER 2, will present a detailed review of the literature outlining the importance of calves to dairy industry; calf growth and development; microbiome development in rumen and small intestine of calves; supplementation strategies available to the farmers; effects of *Macleaya cordata* supplementation to agricultural animals; and current strategies in microbiome analysis. CHAPTER 3 will present materials and methods relative to, health and safety, volatile fatty acid profile, gastrointestinal tract morphology, and rumen and intestinal microbiome analyses. The following chapter, CHAPTER 4, provides results of the study. The final chapter, CHAPTER 5 summarizes the most important findings of the current study and conclusions.

CHAPTER 2. LITERATURE OVERVIEW

The importance of calves in dairy industry

Newborn heifer calves represent the cows that will be in milk in two or more years. Therefore, dairy heifer calves should be managed in a way that ensures they achieve the maximum milk production potential in the future (Zanton and Heinrichs, 2005; Soberon et al., 2012; Van Amburgh et al., 2012). Furthermore, healthy calves with satisfactory growth are extremely important to the economic stability of dairy farms as it helps maintain an optimum herd turnover rate, which usually exceeds 30% per year (Overton et al., 2020). The turnover rate refers to the number of cows culled annually relative to the entire herd size (Sattler et al., 1989). The optimal turnover rate depends on the specific conditions of each farm. A model simulation conducted by Congleton et al. (1984) suggests that a turnover rate of 20% to be the most economically advantageous to most farms. Additionally, enhanced heifer growth helps achieve early weaning and age at first calving; a situation that brings economic benefits (Ettema and Santos, 2004; Krpálková et al., 2014). According to a model simulation conducted by Hawkins et al. (2019), the average cost of raising a calf from birth to weaning can be as costly as $6.19 per day. In comparison, the cost of raising a heifer calf from weaning to calving was much cheaper at $2.40 per day (Tranel, 2019). Therefore, promoting the growth of neonatal calves and thus achieving weaning at an early age can favorably affect the overall cost of production in a dairy operation. Shivley et al. (2018) conducted a survey covering 102 commercial dairy farms in 13 states in the US and found several factors including colostrum quality, diseases, the plane of nutrition, and safety of milk or milk replacer (e.g., pasteurized vs. non-pasteurized milk), housing type, and even the type of bedding significantly influence ADG of pre-weaned dairy calves. Considered together, those factors emphasize three underlying requirements to achieve satisfactory growth for young

calves. Those requirements include 1) suitable genetics, 2) proper nutrition, and 3) sound health (immune) status. Nutrition plays a critical role in determining the growth individually or through its interactions with genetics and the immune status. Some recent developments such as mitigating antibiotic use and increased production of dairy × beef calves demand improved understanding of those interactions.

Significance of neonatal calf nutrition

The critical involvement of colostrum in the growth and health of young calves has been well described (Kertz et al., 2017). The physical form of the diet and plane of nutrition also have been two major considerations in raising neonatal calves. When calves suckle milk or milk replacer, those liquids bypass the rumen and are directly shunted to the abomasum as a result of reflexive closure of the esophageal groove (McGeady et al., 2006). Gastric and intestinal digestion of lactose, fat, and protein provides small- and medium-chain fatty acids, glucose, galactose, and amino acids for the maintenance of vital body functions and the growth during early life (Khan et al., 2011). Several studies have demonstrated that pre-weaning growth is directly proportional to the amount of milk or milk replacer consumed (Khouri and Pickering, 1968; Hodgson, 1971; Huber et al., 1984). A National Animal Health Monitoring System (NAHMS) survey study in 2014 (USDA, 2016) found that 54.5% of pre-weaned dairy calves in the US were fed 6 or more liters of milk or milk replacer per day. This trend of feeding high liquid diet volumes is possibly a result of strong relationships of high liquid diet volumes with the growth and health of calves shown in studies during the last two decades (Soberon et al., 2012). On the other hand, high milk or milk replacer allowances are often related to an insignificant solid feed consumption that would delay rumen development (Wickramasinghe et al., 2019). Unlike milk and milk replacer, solid feed, also known as “starter”, moves into the rumen and provides substrates for rumen fermentation and thus volatile fatty acid (VFA) production, which is critical for accelerating physical, morphological,

microbial, and metabolic development of the rumen (Warner et al., 1956; ; Coverdale et al, 2004; Yohe et al., 2019). In addition to the nutrient composition and digestibility having a direct impact on VFA composition, the physical form (e.g., particle size) of feed present in the rumen also contributes to the overall enhancement of rumen development (Beharka et al., 1998; Coverdale et al., 2004). Paradoxically, rumen development, however, begins even before the consumption of starter is initiated indicating a positive effect of the parenteral supply of nutrients originating from the liquid diet on rumen development (Baldwin et al., 2004; Yohe et al., 2019). Corroborating this idea, the development of the small intestine is positively linked with rumen development in dairy calves (Gorka et al., 2011). Therefore, understanding the development of the rumen in response to a different form of feed (liquid vs. solid) taking a different route (rumen vs. small intestine) of the digestive tract of calves is critical to understand the impact of dietary nutrient modifications and feed additive supplementations on digestive tract development and the growth of calves.

Rumen development in neonatal calves

The digestive tract of ruminants is specialized to effectively digest fiber by having a complex stomach with four distinct compartments namely rumen, reticulum, omasum, and abomasum. The rumen and reticulum together referred to as reticulorumen where >80% microbial digestion and fermentation of fiber takes place (Elsden et al., 1945; Balch, 1950). Nonetheless, at birth, calves have underdeveloped reticulorumen, hereafter called the rumen, which only accounts for one-third of total stomach capacity (Tamate et al., 1962). It is generally considered that rumen development in cattle completes within 6 months of age and the developed rumen represents approximately 80% of the total capacity of the complex stomach (Tamate et al., 1962; Govil et al., 2017). Rumen development can be described as related to three major components including 1) physical and morphological development (e.g., total mass and papillae number and length; Warner et al., 1956), 2) metabolic development (VFA absorption and metabolism in the epithelium;

Stevens et al., 1966) and 3) microbiota community development (Fonty, 1989). Early studies discovered that the "bulk" of feeds stimulated the physical development of the rumen, even when indigestible material such as plastic sponges (Tamate et al., 1962), wood shavings (Smith et al., 1961), and plastic cubes (Hamada et al., 1976) were fed. Those materials, however, did not stimulate the development of papillae, the fingerlike projections of the epithelium that substantially increase the absorptive surface area of the rumen. The failure to stimulate papillae growth by non-nutritive substances suggests a critical role of digestion and fermentation of nutrients in rumen development. Corroborating this idea, Lane and Jesse (1996) showed short-chain fatty acids also known as VFA directly infused into the rumen enhanced rumen papillae development in sheep. Those observations also explained why calves fed a starter at an early age than later had longer papillae in Tamate et al. (1962). As reviewed in Diao et al. (2019), microbiota communities in the rumen also play a critical role in the morphological and metabolic developments of the rumen in calves. Feeding probiotics containing *Bacillus licheniformis*, *Saccharomyces cerevisiae*, and *Megasphaera elsdenii* NCIMB 41125 have been shown to increase papillae development and epithelial metabolism of VFA in the rumen of pre-weaned calves (Muya et al., 2015; Jia et al., 2018). Feed additives, particularly those based on plant extracts can also enhance rumen development in calves (Vakili et al., 2013; Kumar et al., 2018). The direct-fed microorganisms or their products, and plant extracts seem to affect rumen development by modulating the VFA profiles. Rumen VFA profiles characterized by elevated percentages of butyrate and propionate are shown to stimulate the morphological and metabolic developments of the rumen (Baldwin et al., 2004). Interestingly, butyrate supplemented in milk and thus bypassing the rumen had been shown to stimulate rumen papillae development indicating an impact of parentally supplied nutrients on rumen development (Gorka et al., 2009). Moreover, changes in

microbiota composition in the small intestine have been shown to influence the microbiota composition in the rumen of dairy calves (Malmuthuge et al., 2015). Moreover, nutrients in milk or milk replacer can increase the plasma concentration of growth factors such as insulin-like growth factor-1 (IGF-1) that stimulate morphological and metabolic developments of the rumen (Baldwin et al., 1999; Žitňan et al., 2006; Gorka et al., 2011). Taken together, nutrients and feed additives in milk or milk replacers that bypass rumen can, however, influence rumen development by multiple mechanisms. The improvements in rumen development are often postulated to be associated with enhanced growth and starter intake representing two major goals of any dairy calf management program. Roth et al. (2009) investigated the relationships between papillae length in different regions of the rumen and the weight gain of calves. They found that long papillae were associated with high weight gain in calves at 15 wk of age. On the contrary, a study conducted by Kern et al. (2016) found no relationships of papillae length, width, or density with weight gain or starter intake of a cohort of 48 older streets at 49 wk of age. Findings of those studies point out that if one were to manipulate rumen papillae morphology, it must be done when calves are young (e.g., < 5 months of age).

Volatile fatty acid profiles in the rumen of young calves

Short-chain fatty acids that are also known as VFA contain one to seven carbon atoms and exist in both straight- or branched-chain configurations (Bergman, 1990). Ever since the original discovery by Tappeiner in the late 1800's, it has been common knowledge that microorganisms in the rumen and large intestine ferment plant matter into VFA predominantly acetic, propionic, and butyric acids along with methane and carbon dioxide. The VFA produced in the rumen stimulate papillae development (described above), rumen motility (McGilliard et al., 1965), and the capacity to absorb VFA (Sutton et al., 1963). In addition, Anderson et al. (1987) demonstrated the ability of the rumen to ferment nutrients in newborn calves fitted with a rumen cannula at 3 d of age. They

measured total VFA concentration in the rumen ranging from 25 to 75 mM during the first 2-3 weeks. The molar percentage of acetate was the highest (72%), whereas the molar percentage of propionate was the lowest (22%) during that time. The molar percentage of acetate decreased, whereas that of propionate increased as calves consumed increasing amounts of starter. The average molar percentage of acetate and propionate in the rumen at 12 wk of age were 55% and 34%, respectively. Anderson et al. (1987) also observed the presence of branched-chain fatty acids such as isovalarate and isobutyrate in the rumen of neonatal calves. Similar to acetate, the molar percentages of isovalarate and isobutyrate in the rumen decreased significantly from wk 1 to 12. As mentioned above, rumen development is impossible without the presence of VFA fueling epithelial proliferation (Sander et al., 1959). Using data from multiple studies, Bergman (1990) estimated that 65 and 85% of absorbed propionate, and butyrate respectively were metabolized before appearing in portal blood indicating a significant metabolism within the rumen epithelium. Rumen epithelium metabolizes butyrate into beta-hydroxybutyric acid (BHBA) that is eventually absorbed into the blood. Therefore, plasma BHBA concentration is often used as a marker for metabolic development of rumen epithelium in calves. Being a major fuel for the epithelium, butyric acid stimulates proliferation and inhibits apoptosis of rumen epithelium leading to an enhanced papillae development in young ruminants (Lane and Jesse, 1997). Propionate has also shown similar effects in calves. For instance, rumen papillary growth was stimulated in milk-fed calves receiving either propionate or butyrate directly into the rumen in Tamate et al. (1962). However, the effects of butyrate and propionate on rumen epithelium appear to be more indirect than direct as those VFA do not show such effects *in vitro* (Baldwin et al., 1999). As pointed out in Baldwin et al. (2004), the propionate-induced plasma insulin that is capable of stimulating the

proliferation of rumen epithelium can be one mechanism through which VFA would indirectly enhance the papillae development.

Rumen microbial development

Pounden and Hibbs (1948) pioneered studying microbial communities in the rumen of calves. About a decade later, Bryant et al. (1958) isolated cellulolytic bacteria in the rumen of one-week-old calves. Fonty et al. (1987) discovered the presence of methanogens in the rumen of 3 to 4 d old lambs. Overall, those early works showed a reasonable diversity in microbial community composition in the rumen of newborn calves. The robustness of those investigations was, however, limited by the technology of that time. For instance, Pounden and Hibbs (1948) were simply using observations under the microscope, and Bryant et al. (1958) and Fonty et al. (1987) were using basic culturing techniques. Since the development of high throughput technologies such as DNA and RNA sequencing techniques in the late 1980s, scientists have been able to effectively determine the role of microbial community composition in developmental processes, host metabolism, and physiology (Hiergeist et al., 2015). According to studies using new technology, the most dominant bacterial phyla in the rumen of calves are *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Jami et al., 2013). The diet plays a significant role in determining the diversity of microbiota at phyla, genus, as well as species levels in developing rumen. At 2 days of age, *Proteobacteria* is the most dominant phylum, comprising 70.4% of the total population. *Bacteroidetes* and *Prevotella* become the most dominant phylum once starter intake is significant pre-weaning, and once calves are weaned, respectively (Rey et al., 2014).

In addition to diet-related changes, the anatomical location or the ecosystem within the rumen also play important role in determining the microbiota composition. One study demonstrated a significant difference in microbiota community composition between the digesta- and rumen wall-associated bacteria in the rumen of pre-weaned calves (Malmuthuge et al., 2014).

Moreover, scientists have also noted significant variability in bacteria and protozoa communities between the liquid- and solid-associated microbiota in the rumen. For instance, Munch-Peterson and Boundy (1963) demonstrated greater numbers of bacterial colonies in the dorsal sac than the ventral sac of the rumen. Martin et al. (1999) discovered greater fibrolytic enzyme activities in the dorsal sac than the ventral sac of the rumen in cattle. The differences in physiochemical parameters such as pH, osmolality, redox potential, and feed particle size explain a considerable proportion of the intraruminal variability of microbiota composition (Lampila and Poutianen, 1966; Yang and Varga, 1989; Martin et al., 1999).

Intestinal development in neonatal calves

The small intestine (SI), the major site of digestion and absorption of nutrients consists of 3 segments namely the duodenum, jejunum, and ileum. Espe and Cannon (1940) at Iowa State University reported first that the length of SI is seven times the body length in calves. Nonetheless, compared to the length, the finger-like projections of the mucosa known as villi are more powerful determinant of the inner surface area representing digestive and absorptive capacities of SI. As stated in Clevens (2013), villi and the invaginations of the epithelium around villi namely crypts were discovered in the 1700s. Villi consist of terminally differentiated cells, primarily absorptive enterocytes, mucin-secreting goblet cells, and enteroendocrine cells (Boudry et al., 2004). Crypts consist of terminally differentiated Paneth cells that are responsible for secreting lysozyme and other antimicrobial substances (Clevers, 2013). Villi and crypts also contain base columnar stem cells important for the development and repairing of the villi (Leblond and Stevens, 1947). As shown by Toofanian et al. (1976), the development of villi and crypts in the fetus of calves were noted at 30 and 110 d of pregnancy. According to those authors, intestinal mucosa acquires a morphology similar to that of mature animals toward the end of pregnancy. Lyons et al. (2020) reported that there was no difference in villi height between calves at birth and 96 days of age.

However, those authors also noted that several factors including the diet and stresses affect the ability to maintain the morphology of SI during the early stages of development in calves (Lyons et al., 2020).

Observations made on piglets highlight that the pathogenic bacteria, weaning stresses, and certain anti-nutritional factors present in feeds can damage villi. On the other hand, the crypts bearing stem cells needed to repair those damages seem to become deeper in response to those deleterious conditions (Kitt et al., 2001). Therefore, improved villus height: crypt depth ratios (VCR) indicate a lack of prevalence of such conditions or the ability of SI to successfully combat such conditions. The plane of nutrition can influence villus height and crypt depth at different degrees depending on the segments of SI (Schaff et al., 2018) or breed (Gorka et al., 2011; van Keulen, et al., 2020) of calves. A significant interaction between breed and the segments of SI on intestinal morphology has also been observed. In a study evaluating the differences in intestinal morphology between Holstein (dairy) and Charolais (beef) bulls, the beef bulls had longer villi in the duodenum and jejunum, but shorter villi in the ileum than dairy bulls (Zitnan et al., 2008). Overall, as stated above, SI of calves is fairly developed at birth, but the factors such as nutrition, diseases (e.g., scours) and stresses, and their interactions with host genetics can still change the characteristics such as villus height and VCR in young calves. Interestingly, the developmental changes in SI can be associated with similar changes in the rumen. For instance, Gorka et al. (2011) showed that replacing whole milk with milk replacer decreased simultaneously the villus height and rumen papillae length in pre-weaned dairy calves.

Microbial development in the lower gut of calves

H.W. Smith pioneered in describing microbiota community composition in SI of calves. He isolated bacteria from SI of newborn calves, and observed *Escherichia coli*, *Clostridium welchii* and *Streptococci* had already been established in SI. The establishment of *Lactobacilli* was

found 2 days after calves had been born (Smith, 1965). The majority of studies conducted later, particularly those in the 1980s, were focused primarily on *E. coli* as a result of its significance in the development of diarrhea in neonatal calves (Hall et al., 1985; Chanter et al., 1986). A survey conducted by the USDA shows that nearly 24% of dairy calves in the US develop diarrhea and 74% of those caves are treated with antibiotics (USDA, 2010). The roles of the rest of the microbial communities in SI of calves and their responses to nutrition and other management practices are poorly understood. As a result of the difficulties in collecting samples from SI of live animals, most of the studies aiming at understanding microbiota composition in SI have used the fecal microbiome as a proxy.

The fecal microbiome analyses available in the literature demonstrate age-dependent shifts in the lower gut microbiota composition of calves, which were similar to the changes in the rumen. For instance, *Lactobacillus, Enterococcus, Bifidobacterium,* and *Enterobacteriaceae* were the most abundant genera in neonatal calves. However, those genera were no longer detectable at 9 months of age, when *Bacteroides* and *Prevotella* were the most abundant (Uyeno et al., 2010). At the phylum level, *Firmicutes* was the most dominant representing 63.8% to 81.9% of species in the feces. The other dominant phyla are *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria*. Malmuthuge et al. (2014) studied digesta- and mucosa-associated microbiota composition in different segments of SI in slaughtered calves. The authors found that mucosa-associated bacteria were more diverse than the digesta-associated bacteria in both small and large intestines. The authors attributed this greater diversity to some roles of mucosa-associated bacteria in host immune system (Malmuthuge et al., 2014). Even though the interactions between gut microbiota and the host can affect the health and nutrition in humans (Krajmalnik-Brown et al., 2012), such associations are yet to be elucidated for calves. Moreover, the responses of microbiota

in SI to dietary nutrient composition and novel feed additives having antimicrobial properties are poorly understood for calves. On the other hand, current technologies such as 16S rRNA gene sequencing would help in exploring such responses rather efficiently if the collection of representative samples from SI is possible.

16S rRNA gene sequencing to analyze microbiota diversity

As mentioned earlier in this review, new technologies such as 16S rRNA gene sequencing have made gut microbiota analysis rather efficient, robust, and high throughput. Early investigations relying on traditional isolation and culturing methods were limited to a very small population of gut microbiota including aerobic bacteria as applying those methods with anaerobic bacteria was challenging (Sarangi et al., 2019). There are several important reasons why 16S rRNA gene sequencing is the most widely used technique for microbiome analyses and is even referred to as a "gold standard for microbiome analysis" by several scientists (Kolbert and Persing, 1999; Bouchet et al., 2008; Sarangi et al., 2019). The 16S rRNA gene in prokaryotes such as bacteria and archaea are about 1600 base pairs long (Gutell, 1992) and is comprised of both highly variable as well as conserved regions (Maidak et al., 1997). Within the 16S rRNA gene, 9 hypervariable regions represent a significant evolutionary divergence. Therefore, those regions help differentiate closely related bacterial species. The conserved regions, on the other hand, assist in identifying the higher-ranking taxa (e.g., phylum) of those bacterial species (Sarangi, 2019). However, a review article by Rizal et al. (2020) describes several limitations of 16S rRNA gene sequencing. On average 300-350 pb are used for the analysis which is not sufficient to differentiate between some species. Additionally, 16S rRNA gene sequencing does not discriminate between DNA that comes from living and functioning bacteria and dead inactive species, making it difficult to interpret their function. Greatest issue outline by Rizal and colleagues is the resolution of the 16s rRNA region. The bioinformatics analysis of the 16S rRNA gene sequencing data is often used to determine the

alpha diversity that describes primarily the richness (e.g., the total number) of bacterial species and the evenness of their abundance in a given ecosystem or a habitat (Hagerty et al., 2019). The Shannon index (Shannon and Weaver, 1949) and Simpson index (Somerfield et al., 2008) are two popular parameters often included in alpha diversity analyses. The Shannon index is determined considering primarily the species richness. Therefore, the Shannon index value increases as diversity of a bacterial population increases. The Simpson index is a measure of the probability that two individuals randomly selected from a sample will belong to the same species. Therefore, a high Simpson index value indicates low biodiversity in a sample.

Phytogenic feed additives for calves

Concerning the positive impact of the gastrointestinal tract development on the overall maturation of calves, many feed additives for calves are aimed at improving gut health. Among those supplements, antibiotics have been predominant. Despite the growing concerns about antibiotic use in animal agriculture, numerous studies demonstrate significant improvements in the health and growth of calves for antibiotic supplements (Donovan et al., 2002; Heinrichs et al., 2003; Hossani et al., 2010). The major concern of antibiotic use in animal agriculture is its contribution to antibiotic resistance developed by microbiota in animals that would be harmful to humans. Wichmann et al. (2014) point out a potential transfer of antibiotic-resistant bacteria to humans through manure that is used to fertilize crop fields. However, it is important to note that a comprehensive report compiled by USDA indicates that antibiotics, typically used in animal agriculture are not commonly used in humans and therefore do not contribute to human antibiotic resistance (Hurd et al., 2004). In many European countries and the US, the use of antibiotics as feed additives in animal agriculture is currently prohibited (USDHHS, 2010). Therefore, the development of feed additives alternative to antibiotics is of great interest in animal feed industries around the world. In a review article, Gadde et al. (2013) identified probiotics, organic acids,

prebiotics, symbiotic, enzymes, antimicrobial peptides, bacteriophages, clay, metals, and plant extract as potential alternatives to the antibiotics. Feed additives based on plant extracts, hereafter called phytogenic feed additives, are found to possess antimicrobial properties as they contain secondary metabolites of plants, produced as a part of plants' defense mechanisms against deleterious microorganisms (Rossow et al., 2020). Moreover, those phytogenic feed additives attract high consumer acceptance as they are considered "natural". In a review article, Yang et al. (2015) acknowledges antimicrobial, anti-inflammatory, and antioxidant properties of the phytogenic feed additives that can improve the health, wellbeing, and productivity of livestock. There are several modes of action by which the antimicrobial action of the phytogenic feed additives is achieved. Some phytochemicals, like those present in the *Melaleuca alternifolia* commonly known as tea tree, can cause leakage of the lipid membranes of bacteria, ultimately causing death (Carson et al., 2002). Other plants contain phenolic compounds that can also have antimicrobial effects (Tranter et al., 1993). Lillehoj et al. (2018) reviewed the effects of several phytogenic feed additives and highlighted immunomodulatory and tumor suppression actions in poultry. Nonetheless, a large part of current information about phytogenic feed additives is limited to laboratory animals, pigs, and poultry. Given that the rumen is a crucial modifier of many feed additives, the effects of phytogenic feed additives found in other species must be extrapolated cautiously to ruminants.

Macleaya cordata plant extract and the alkaloids it contains

Macleaya cordata, also known as plum poppy or tree celandine, is a plant that belongs to the *Papaveraceae* family (the poppy family) and is native to China and Japan. In the US, the plant is present mostly in Eastern states (e.g., east of the Mississippi river). *Macleaya cordata* has been widely used for medicinal purposes in China for centuries. In a review article, Dvorak et al. (2006) attributes the medicinal properties of *Macleaya cordata* to alkaloid compounds found primarily in

its fruits. Those authors acknowledge sanguinarine and chelerythrine as the major and most-studied alkaloids, even though Qing et al. (2014) identified 21 different alkaloids present in *Macleaya cordata*. Roberts and Wink (1983) define alkaloids as "cyclic compounds that contain nitrogen in a negative oxidation state which are limited to distribution in living organisms". In the past, alkaloids were used for a variety of purposes in human medicine. Some examples include pain medications, cancer treatments, and local anesthetics (Schläger and Dräger, 2016). Sanguinarine and chelerythrine are classified as isoquinoline plant alkaloids as they are derived from a compound namely isoquinoline (Figure 1). Both sanguinarine and chelerythrine can cross the cell membrane as hydrophobic neutral molecules. However, once inside the cells, they are converted into biologically active cationic forms (Dvorak et al., 2006). Due to the diverse medicinal effects (e.g., anti-inflammatory and antioxidant) of sanguinarine and chelerythrine in humans and laboratory animals (Zdařilová et al., 2006), the *Macleaya cordata* plant extract (MCE) has been investigated as a feed additive that could improve the health and production of livestock species. The majority of those investigations are, however, focused on monogastric species.

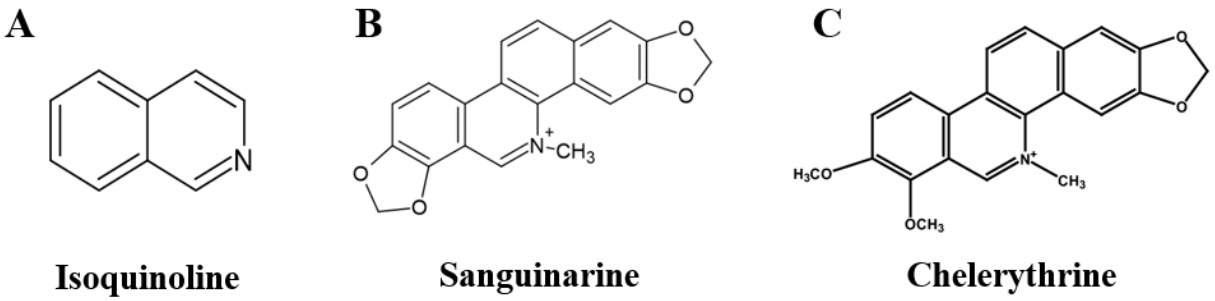

Figure 1. Isoquinoline (A), the parental compound for plant alkaloids, sanguinarine (B), and chelerythrine (C)

Macleaya cordata plant extract (MCE)-based feed additives in pigs and poultry

Khadem et al. (2014) studied the anti-inflammatory effects of MCE on the health and growth of chickens both *in vivo* and *in vitro*. The *in vitro* experiments demonstrated a significant

anti-inflammatory action of MCE that inhibited the expression of several important genes responsible for inflammation in the jejunum. In *in vivo* experiments, chickens supplemented with MCE in drinking water (25 to 100 mg/L) had greater body weights and feed conversion efficiency than those without an MCE supplement (Khadem et al., 2014). On the contrary, in a study conducted by Juskiewicz et al. (2011), chickens supplemented with 15 mg/kg of MCE delivered in feed for 5 weeks did not affect feed efficiency or body weight when compared to the control group throughout the study. It is important to note that Khadem et al. (2014) had not been able to report the actual intake of MCE as a result of significant spillage of drinking water. When piglets were supplemented with two doses of MCE (15 or 50 mg/kg of the diet) for 48 days, those with 50 mg of MCE/kg achieved greater body weight and consumed more feed than the control and 15 mg/kg groups (Kantas et al., 2014). Chen et al. (2018) also observed similar results in piglets with the same dose (50 mg/kg) of MCE. The MCE appears to improve growth and feed conversion efficiency at least partly by ameliorating the severity of common illnesses of young animals. In Liu et al. (2016), a supplementation of MCE at 40 mg/kg for 28 days decreased scours scores of piglets to the level that was achieved with antibiotic (0.2 g of colistin/kg of feed). Moreover, piglets supplemented with MCE had taller villi in the ileum, shallower crypts in the jejunum, and greater VCR in both jejunum and ileum. Moreover, Liu et al. (2016) found significant changes in microbiota composition in the intestines of growing piglets consuming an MCE supplement in the diet. For instance, supplementation of MCE increased the abundance of favorable bacteria, *Lactobacillus* and *Bifidobacterium* in the caecum, whereas it decreased the abundance of pathogenic bacteria, *E. coli* and *Salmonella* in the ileum.

Supplementing MCE in ruminant animals

Even though the number of studies focused on ruminants is less than that of monogastric animals, existing data helps develop a reasonable understanding of the overall impact of MCE in

ruminant species. A study conducted by Aguilar-Hernández et al. (2016) found that steers supplemented with graded levels of MCE (0, 2, 4, and 6g/head/d) in the diet significantly affected the nitrogen utilization efficiency. High doses of MCE decreased ammonia concentration in the rumen and increased microbial protein production. High doses of MCE also increased postruminal protein digestion leading to high total tract digestibility of protein. In another study, dietary supplementation of MCE decreased inflammation of rumen mucosa in beef steers fed a high-grain diet (Sanches et al., 2020). In uncastrated male lambs, supplementation of MCE at 320 mg/kg of DM resulted in increased papillae number per cm^2 (Lima, et al., 2019). Based on the literature described previously in this chapter, such an improvement in rumen morphology would be advantageous to young calves. Even though it is comprehensively described for monogastric animals, the impact of MCE on microbiota composition in the rumen and the small intestine is poorly understood for ruminant animals. Based on an *in vitro* experiment, Petri et al. (2019) suggests potential effects of MCE on bacterial communities in the rumen. Considering the effects shown in monogastric animals and previously discussed associations between the microbiota in the rumen and the small intestine, MCE can be assumed to also influence microbiota composition in the small intestine of calves. Nonetheless, no study in the literature reports an improvement in growth for MCE supplements in ruminant animals. Toprak (2020) points out the importance of feeding multiple doses for successfully capturing the true effect of MCE on growth. Feeding graded levels of MCE also help evaluate its effect on the health of the animals as well as the safety of food (e.g., meat) they produce (USFDA, 2020). Blood chemistry and hematology analyses are two commonly used assessments of the impact of novel feed additives on the health (e.g., tissue and organ health) of the animals. Determining the residue concentrations of active ingredients of MCE such as sanguinarine and chelerythrine in edible tissues and organs is typically used to assess

the safety of MCE for meat-producing animals (Zhao et al., 2017). Therefore, besides examining the effects of MCE on gut morphology and gut microbiota composition of calves, the experiment described in the next chapter was aimed at evaluating graded levels of MCE in the diet on feed intake, average daily gain, the health, and concentrations of sanguinarine and chelerythrine residues in organs and tissues of calves.

CHAPTER 3. EFFECTS OF A *MACLEAYA CORDATA* EXTRACT PREPARATION ON GROWTH, HEALTH, GUT MORPHOLOGY AND GUT MICROBIOTA COMPOSITION OF CALVES

Animals and treatments

All animal-related procedures in this study were conducted under the approval of the Animal Care and Use Committee at Iowa State University (IACUC-19-204). The trial was conducted at the Dairy Research and Teaching Unit at Iowa State University. Forty dairy (Holstein or Montbéliarde) × beef (Angus or Angus-Simmental) crossbred neonatal calves (20 male and 20 female) of 5±2 d of age were used. All calves used in this study were purchased from a commercial farm (Milk Unlimited, Atlantic, IA) known to have a standardized colostrum feeding program (e.g., feeding colostrum at 10-12% of birthweight during the first 8 h of life). Once calves arrived at the experimental location, ear-notch samples were collected for bovine viral diarrhea (BVD) testing by two veterinarians at Iowa State University. Based on the test results, all calves were found to be free of BVD (data not shown). Calves were housed individually in outdoor calf hutches (2.4m × 1.4m × 1.3m) bedded with straw. After two days of acclimatization to the new housing environment, calves were matched for sex, age, and baseline body weight and assigned to one of four graded doses (CTL, LW, MD, and HG) of two commercial preparations of MCE (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany); 1) Sangrovit Stat Pak® and 2) SangrovitG premix® that were fed to calves during pre- and post-weaning periods, respectively. The doses of each MCE preparation in the corresponding period are given in Table 1.

Feeding and general management

***Pre-weaning*:** Each calf had free access to clean water and a starter feed (Farmers Win Coop, Houston, MN) offered in two separate buckets. Each calf was bottle-fed daily with 6.0 kg of liquid milk replacer (12% solid) at two feedings (3.0 kg per feeding at 0730 and 1930 h). Calves

were weaned partially at 42 d of age by eliminating the morning milk feeding. All the calves then received only 3.0 kg/d liquid milk replacer until they were weaned completely at 49 d of age. At a given feeding time, one-half of each pre-weighted MCE (SangrovitG premix®) dose (Table 1) was mixed separately with 3.6 kg of dry milk replacer. Each dry mixture (3.6 kg) was then reconstituted with 27.0 kg of lukewarm water (40 to 45°C) to prepare a liquid milk replacer to feed calves (n = 10) in each treatment. In addition to the MCE dose, 10g of a coccidiostat containing decoquinate (Deccox-M®, Zoetis, Parsippany-Troy Hills, NJ) was added to the dry milk replacer mix until calves were 28 d of age. The weights of liquid milk replacer offered to and leftover by individual calves was recorded daily.

***Post-weaning*:** Once calves were completely weaned, the MCE preparation was changed from Sangrovit Stat Pak® to SangrovitG premix®. Corresponding doses of the new preparation (Table 1) were pre-weighed into plastic bags and stored at 4°C until being used. Each day in the morning, starter feed was measured into the feed buckets (110% of previous day intake), Sangrovit® was top-dressed on feed and offered to each calf. To prevent the loss of treatment due to the wind and animals sorting, coughing, or exhaling, the dose was gently mixed into the topmost layer (about one inch deep) of feed before the feed was offered. The amounts of starter feed offered, and leftover was recorded daily. The ingredient composition of the starter feed is given in Table 2. Multiple batches of starter feed were purchased during the course of the study. Representative feed samples of each batch were analyzed for nutrient composition using the standard procedures in Cumberland valley Analytical Services (Waynesboro, PA). The average nutrient composition across the feed batches is given in Table 2.

Blood analyses and body weight measurements

Before assigning to the treatment, the body weight (BW) of individual animals was recorded. Following BW measurements, blood was drawn from the jugular vein into two

vacutainer tubes one with sodium-citrate and the other with EDTA for analyses of prothrombin and fibrinogen, and hematology parameters, respectively. An additional blood sample was collected into a vacutainer tube without anticoagulants for serum chemistry analysis. The BW measurements and blood sample collection were repeated at 45 and 90 d of age. At each time, blood in EDTA tubes was stored at 4°C until analyzed. Blood in the other tubes was centrifuged at 4000 rpm and 4°C for 15 min to separate serum or plasma, which were then stored at -20°C until being analyzed.

All blood analyses were carried out according to good laboratory practices (GLP) guidelines in Quality Vet Laboratory (Davis, CA). This laboratory provides testing services for GLP-regulated (21CFR Part 58 compliant) trials in areas of hematology, blood chemistry, and blood coagulation. Hematology including red and white blood cell counts, hemoglobin, platelets, prothrombin, fibrinogen, and corpuscular volume were analyzed with ADIVA 120 system (Siemens Healthneers, Erlangen, Germany) and STA Compact Max® coagulation analysis system (Diagnostic Stago Inc., Parsippany, NJ). The serum chemistry analysis included total protein and nitrogen, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, γ-glutamyltransferase, creatinine and creatine kinase, amylase, total bilirubin, and minerals (Ca, P, Na, K, Cl, and Mg). Those analyses were carried out using an AU400e Chemistry analyzer (Beckman Coulter, Brea, CA).

Tissue and digesta sample collection

At the end of the study (d 90), calves were matched for age and treatment and assigned to four groups (10 calves per group) each of which was euthanized on one of four consecutive days. That arrangement led to similar ages of calves at euthanasia (95±2 d). Calves were transported to the livestock infectious disease isolation facility (LIDIF) at Iowa State University (Ames, IA) for euthanasia that was started at 0930 h. A licensed veterinarian performed stunning with a captive

bolt pistol followed by immediate exsanguination. The same veterinarian assisted in opening the body cavities for tissue collection. About 50g samples of the liver (cross-section of each lobe), kidney (from each kidney), muscle (loin, flank, and hind leg separately), and fat (subcutaneous, mesenteric, and perirenal separately) were collected. All samples were snap-frozen immediately in liquid nitrogen, transported in dry ice, and stored at -80°C until they were sent to ATC Scientific (North Little Rock, AR) for sanguinarine and chelerythrine residue concentration analyses. About 300g of rumen content from the ventral sac and about 50g of digesta from the jejunum were also snap-frozen and stored at -80°C. Additionally, tissue samples of the rumen (ventral sac), duodenum, jejunum (15 and 80 cm distal to pyloric sphincter, respectively), and ileum (15 cm proximal to ileocecal junction) were obtained and saved in a 10% formaldehyde solution for gut morphology analysis. Euthanasia and sample collection were completed within about 3.5 h on each day.

Histological analysis of rumen and intestinal mucosa

Because only the highest dose of MCE (HG) tended to be associated with greater nutrient intake relative to CTL, the rumen, duodenum, jejunum, and ileum tissue samples of only CTL and the highest dose of MCE (HG) were used in this analysis. Tissue cross-section preparation and imaging were performed in the Veterinary Diagnostic Laboratory at Iowa State University (Ames, IA). The samples fixed in 10% formaldehyde were grossed and loaded into plastic cassettes, placed into the tissue processor, and left overnight for dehydration and infiltration with paraffin. The next day, tissues were embedded in paraffin blocks and stained with hematoxylin and eosin. The images of tissue cross-sections were obtained at 10× magnification using an Olympus DP73 camera mounted onto an Olympus B53 microscope (Olympus Corporation, Shinjuku City, Tokyo, Japan). Papillae length and width, villus height, or crypt depth in three non-overlapping fields of each

tissue cross-section were measured using the ImageJ software (National Institutes of Health, Bethesda, MD).

Rumen VFA analysis

Volatile fatty acid (VFA) concentration in the rumen were analyzed using the fluid (~15 mL) collected by squeezing rumen content samples through four layers of cheesecloths. The solids and approximately 5 mL of liquid were stored at -80°C for forthcoming microbiota analyses. The remaining liquid was centrifuged at 4°C and 9,000 rpm, for 15 min. Then, 5 mL of the supernatant was mixed with 1.0 mL of 25% metaphosphoric acid and centrifuged again for 10 min at 4000 rpm at room temperature and the supernatant was separated. Solutions of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, each at 1:1, 1:2, 1:4, and 1:6 dilutions were prepared using deionized water and used as the standards. 100µL of 2-ethyl butyric acid was added as an internal standard to all samples as well as the standards. Concentrations of VFA in samples were analyzed using Varian CP-3800 Gas Chromatograph (Varian Medical Systems, Palo Alto, CA).

DNA extraction

The DNA was extracted from approximately 0.25 g of each rumen liquid and solid, and jejunal digesta and mucosa samples using Qiagen DNeasy Powersoil Powerlyzer Kit® (Qiagen Sciences Inc, Germantown, MD), following the protocol provided by the manufacturer. The process included a bead-beating step for 1 minute at 22°C using Fisherbrand™ Bead Mil 24 Homogenizer (Fisher Scientific, Portsmouth, NH) for mechanical lysis of the cells. The concentration of DNA in cell lysis was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and the concentrations were adjusted to 25 to 50 ng of DNA/µL using PCR-grade water. Samples were loaded on a 96-well Microtiter™ plate (ThermoFisher Scientific, Waltham, MA) and sealed with Nunc™ sealing tape (ThermoFisher

Scientific, Waltham, MA). All samples were stored at -80°C until they were submitted to Iowa State University DNA Facility (Ames, IA) for sequencing.

16S rRNA gene sequencing

DNA sequencing was conducted using the protocol designed to amplify the 16S rRNA genes of bacteria and archaea, described originally by the Earth Microbiome project with some modifications. Template DNA from each sample (one replicate per sample) was amplified using the Platinum™ Hot Start PCR Master Mix (2x) (Thermo Fisher Scientific, Waltham, MA) with the 515F forward-barcoded primer (GTGYCAGCMGCCGCGGTAA; Prada et al., 2015) and the 806R reverse primer (GGACTACNVGGGTWTCTAAT; Apprill et al., 2015). All samples underwent an initial denaturation step at 94°C for 2 min, followed by 45 s of denaturation at 94°C, 20 s of annealing at 50°C, and 90 s of extension at 72°C that were repeated 35 times. Amplicons from each sample were run on an agarose gel with an expected band size ranging from 300 to 350 bp. Amplicon pools were cleaned using Qiagen's QIAquick PCR Purification Kit (Qiagen Sciences Inc, Germantown, MD). Raw sequencing data was saved in fastq format and analyzed using the mother software (version1.43.0, Schloss et al., 2009). The number of raw reads per sample varied from 13,742 to 473, 268. All reads were subjected to quality control using the *screen.seqs* command that removes ambiguous bases and homopolymers greater than eight bases in length. A total of 2606 OTUs in jejunal content, 1984 OTUs in jejunal mucosa, 2761 OTUs in rumen solid and 2895 OTUs in rumen liquid samples passed the quality control steps. Sequences were aligned against the SILVA database (version 138; Pruesse et al., 2012) using the *align.seqs* command and chimeras were removed using the *chimera.vsearch* and *remove.seqs* commands. Alpha diversity parameters including Chao 1, Shannon, Simpson, and inverse-Simpson indices were analyzed using the *summary.single* command in mothur v.1.43.0. Beta diversity was analyzed using the Adonis and beta-dispersion tests in mothur.

Calculations and statistical analysis

The DMI during the pre-weaning was calculated by adding dry matter from the milk replacer and dry matter from the starter feed. The ADG was calculated taking the difference between two consecutive BW measurements which was then divided by the time interval in days. Feed conversion efficiency was calculated taking the ratio between the ADG and average DMI (ADG: DMI) during corresponding time interval. The length and width of rumen papillae and villus height and crypt depth of the small intestine were averaged across the three fields of each tissue image representing a sample from a single animal. The villus height was divided by corresponding crypt depth to calculate villus height to crypt depth ratio (VCR).

Because starter intake, milk replacer intake, DMI had multiple observations made on a single animal, the treatment effects on those variables in pre- and post-weaning periods were analyzed separately with the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) using the following statistical model.

$$Y_{ijklm} = \mu + T_i + W_j + S_k + B_l + (T \times S)_{ik} + C_{ijklm} + e_{ijklm}$$

where, Y_{ijklm} = the response variable of interest, μ = overall mean, T_i = fixed effect of treatment (i = CTL, LW, MD, and HG), W_j = fixed effect of age in weeks, S_k = fixed effect of sex, B_l = fixed effect of breed, $(T \times S)_{ik}$ = fixed interaction effect between treatment and sex, C_{ijklm} = random effect of calf, and e_{ijklm} = random residual error. Body weight, ADG, ADG: DMI, blood chemistry, and hematology having one observation for each calf in a given period were analyzed using GLM procedure of SAS with above model excluding the fixed effect of week and random effect of the calf. When analyzing BW, the baseline BW measurement was used as a covariate. Likewise, when analyzing blood chemistry and hematology, corresponding baseline values were used as covariates. The association of MCE dose with each response variable was tested using linear, quadratic, and cubic orthogonal contrasts linked to both models.

Treatment effects on gut morphology, absolute concentrations of VFA, molar percentages of VFA, and relative abundance of the 100 most abundant OTU and genera in the rumen and jejunum were analyzed with following model using the GLM procedure of SAS 9.4.

$$Y_{ijk} = \mu + T_i + S_j + B_k + e_{ijk}$$

where, Y_{ijk} = the response variable of interest, μ = overall mean, T_i = fixed effect of treatment (i = CTL and HG), S_j = fixed effect of sex, B_k = fixed effect of breed, and e_{ijk} = random error. The Pearson's correlation coefficients between the relative abundance of microbiota taxa and DMI, ADG, rumen papillae length, villus height, crypt depth, and VCR were determined the CORR procedure of SAS 9.4.

CHAPTER 4. RESULTS AND DISCUSSION

Dose-dependent effects of MCE on feed intake and growth

Examining the effects of MCE dose on feed intake and the growth was one objective of the present study as nutrient intake is a major determinant of gut development positively influencing the overall growth and development of calves (Tamate et al., 1962; Baldwin et al., 1999; Roth et al., 2009). The dose dependent effects of MCE on feed intake and the growth during pre-weaning (until 49 d of age) and post-weaning (49 to 95 d of age) periods were analyzed separately as two different commercial preparations of MCE were fed in those two periods. Moreover, the fact that MCE provided in milk replacer bypasses the rumen, whereas MCE supplemented in starter enters the rumen justifies further analyzing the effects during pre- and post-weaning periods separately. The results are presented in Table 3. During the pre-weaning period, MR intake had a quadratic relationship with MCE dose ($P < 0.001$). Milk replacer intake of LW was greater than CTL ($P < 0.001$) and MD ($P = 0.039$) but not significantly different from HG ($P > 0.100$). Nonetheless, regardless of the dose, MCE-supplemented calves consumed more MR than that of CTL ($P < 0.010$, data not shown). Previous studies demonstrated that increased milk replacer intake was related to improved gut development and weight gain in calves (Khouri and Pickering, 1968; Hodgson, 1971; Huber et al., 1984; Górka et al., 2011). Starter intake also had a cubic relationship with MCE dose during the pre-weaning period ($P < 0.001$). The relationship with starter intake was, however, opposite to the relationship with milk replacer intake suggesting starter intake was compensatory to milk replacer intake to some extent. For instance, LW having greater MR intake had lower starter intake compared to CTL and MD ($P < 0.001$). Dry matter intake, which is the sum of DM from the milk replacer and the starter also had a cubic relationship ($P < 0.001$) with the MCE dose. Owing to the greater contribution of starter DM to DMI relative to DM in MR, the

relationship was similar to the relationship of starter intake. Dry matter intake of LW was lower than both CTL and MD ($P < 0.010$) and DMI of HG tended to be greater than CTL ($P = 0.066$). Nonetheless, MCE did not affect ADG or feed efficiency (ADG: DMI) of pre-weaned calves (Table 3).

During the post-weaning period, LW continued to consume less starter compared to CTL ($P = 0.009$), MD ($P = 0.044$), and HG ($P = 0.021$). Consequently, the relationship between DMI and the dose of MCE continued to be cubic ($P = 0.013$, Table 3). Despite the changes in DMI, ADG, or ADG: DMI remained unaffected by MCE ($P = 0.680$). It is noteworthy that a common ADG was calculated for the whole post-weaning period simply by taking the difference between BW measurements at 49 d and 95 d of age. Perhaps, more frequent BW measurements could have provided a better idea about the effects on ADG and ADG: DMI. Nonetheless, previous research (Wang et al., 2017; Michels et al., 2018; Zhang et al., 2019; Lima et al., 2020) do not show an impact of dietary supplements of MCE on ADG of ruminants. Despite the lack of an effect on the overall growth, MCE supplementation seems to influence individual tissue development and carcass composition of cattle (Michels et al., 2018). Considering together, a more detailed assessment would have helped capture the true effects of MCE on the growth and development of calves.

Dose-dependent effects of MCE on the blood chemistry

Phytogenic feed additives including MCE are rich in secondary metabolites produced as a part of plants' defense mechanisms not only against pathogens but also herbivores (Rossow et al., 2020). Therefore, one objective of the present study was to examine blood markers reflecting any deleterious effect on tissues and organs of calves fed graded levels of MCE. The effect of MCE on those blood markers at 49 and 95 d of age marking the end of the pre- and post-weaning periods are presented in Tables 4 and 5, respectively. It is important to note that the blood chemistry of

calves is different from adult cattle for many variables. A limited number of research articles report the blood chemistry of calves, but the observations seem to be quite consistent across the reports (Klinkon and Ježek, 2012). Enzymes such as alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT), and creatinine kinase (CK) are sensitive indicators of organ or tissue damage. None of those enzymes except AST and LDG were affected by MCE doses in both pre- and post-weaning periods ($P > 0.050$). The MCE dose had cubic relationships with the post-weaning concentrations of AST ($P = 0.034$) and LDH ($P = 0.004$). The concentration of AST decreased for LW and MD but then increased for HG. Regardless of the dose, however, mean AST or LDH of MCE-supplemented calves was not different from that of CTL (data not shown). Moreover, the concentrations of ALT, AST, LDH, and CK increased from pre- to post-weaning ($P < 0.001$, data not shown). Previous studies (Knowles et al., 2000; Mohri et al., 2007; Ježek, 2007; Zhang et al., 2019; Zhang et al., 2019) report similar changes validating the measurements of the present study. The MCE dose did not change albumin, total protein, and urea nitrogen concentrations in both pre- and post-weaning periods. The creatinine ($P = 0.042$) and total bilirubin ($P = 0.047$) concentrations had cubic relationships with MCE dose in pre-weaned calves. Elevated blood creatinine concentrations are usually indicative of an impairment of kidney functions such as the glomerular filtration rate. The LW and HG increased the creatinine concentration (0.95-0.96 vs. 0.84 mg/dL) while MD did not change it relative to CTL. Yu et al. (2019) established the reference intervals for several serum chemistry parameters using data from 134 healthy calves at 49 d of age, the same age of pre-weaned calves providing blood chemistry in the present study. The authors reported a reference interval of 0.43 to 1.03 mg/dL for blood creatinine in healthy calves. Referring to that interval, 0.95 to 0.96 mg/dL concentrations would not alarm a deleterious

effect of MCE dose. On the other hand, Montanholi et al. (2017) report a positive relationship between feed efficiency and blood creatinine concentration in beef steers. Moreover, the creatinine elevated within healthy limits can also indicate an increased muscle mass. Istasse et al. (1990) showed a positive correlation of plasma creatinine with dressing percentage and the proportion of lean meat in the carcass. Total bilirubin including both conjugated and albumin-bound forms result mainly from the breakdown of hemoglobin (Doornenbal et al., 1988). Pre-weaned calves receiving medium and high doses had lower total bilirubin concentrations than CTL while the concentration of LW was similar to CTL. The concentrations were, however, within the reference ranges for healthy calves indicating no deleterious effect such as extravascular hemolysis, liver damage, or anemia for MCE supplementation (Tennant, 1997). Minerals such as calcium, inorganic phosphorous, sodium, chloride (Cl^-), and magnesium play an important role in mineral homeostasis across body fluids, and bone, muscles, and nerve tissues. The concentration of none of those minerals except Cl^- responded significantly to the MCE dose. The concentration of Cl^- increased linearly with MCE post-weaning ($P = 0.003$). Referring to the range of healthy cows (96 to 109 mEq/L, Latimer et al., 2003) the lower concentrations associated with MCE seem to still represent healthy cattle. Plasma glucose and cholesterol did not change with the MCE dose.

Dose-dependent effects of MCE on the hematology

The effects of MCE dose on major parameters of hematology analysis performed at 49 (pre-weaning) and 95 d of age (post-weaning) are given in Tables 6 and 7, respectively. The hematology analysis is a useful tool that helps investigators assess potential blood disorders and damages to tissues or organs. Panousis et al. (2018) provide reference ranges for some hematology parameters of healthy neonatal calves using data from a large calf population (n = 254). Moreover, Brun-Hansen et al. (2006) reported reference ranges for healthy calves up to 6 months of age, but the sample size was smaller (n = 15). The MCE dose did not affect the majority of hematology

parameters that were within the reference ranges of healthy calves published by the aforementioned authors. The relationship between the MCE dose and red cell distribution width (RDW) was cubic ($P = 0.048$) as LW and MD had lower values, while MD having a similar value relative to CTL pre-weaning. The RDW is indicative of the oxygen-carrying capacity of red blood cells and thus low RDW could indicate anemia. Nonetheless, the differences do not seem to bear a biological significance as RDW of all the treatments falls within the ranges of healthy calves. MCE dose did not affect red blood cell count (RBC), hemoglobin concentration, or mean corpuscular volume (MCV) in both periods ($P > 0.050$). Hemoglobin concentration (11.80 vs 12.85 g/dL) and MCV (34.26 vs 35.89 fL) increased ($P < 0.001$) from pre-weaning (~ 7 wk of age) to post-weaning (~ 13 wk of age). Nonetheless, the RBC, hemoglobin concentration, and MCV were within the ranges of healthy calves at 6 to 16 wk of age (Brun-Hansen et al., 2006). Moreover, the absolute monocyte counts decreased linearly with the MCE dose post-weaning ($P = 0.047$). High monocyte counts are observed in cattle undergoing stresses or suffering from infections (Roland et al., 2014). Perhaps, feeding MCE helps calves cope with stress and infections.

Effects of MCE on gut morphology and rumen VFA

The effect of MCE supplemented at the highest dose (HG) on the morphology of papillae in the rumen and the mucosa of the small intestine are presented in Table 8. As observed previously for weaned pigs (Chen et al., 2019), HG increased villus height and villus height: crypt depth (VCR) in all three regions of the small intestine ($P < 0.070$). Moreover, the crypt depth of HG was lower than that of CTL in the jejunum ($P = 0.033$). Taller villi represent an increased availability of brush-border enzymes for digestion and an increased surface area for the absorption of nutrients. Deeper crypts can indicate inflammations in the epithelium. Moreover, VCR is found to be associated positively with the barrier function of the small intestine that prevents the luminal pathogens and their toxins from translocating to the bloodstream (Van Der Hulst et al., 1998).

Overall, the increased villus height and VCR highlight improved nutrient availability and gut health for MCE that can promote the growth of calves. The similar ADG and ADG: DMI between CTL and HG (Table 3), however, do not support such a notion. Perhaps, as mentioned above, a more detailed assessment of growth could have help capture true associations of MCE with growth in the present study. Moreover, it is noteworthy that calves in the present study had zero mortality and no severe cases of common health issues such as diarrhea. Therefore, the effects of MCE could have been more prominent in calves with such health issues. The supplementation of MCE also increased papillae length in the rumen (P = 0.013) suggesting the increased surface area for the absorption of VFA produced in the rumen. Consistently, Lima et al. (2020) showed an increased surface area of rumen papillae in sheep for an MCE supplement. The effects of MCE on gut morphology that were consistent between the rumen and the small intestine agree with Górka et al. (2011) demonstrating a positive association between the development of the rumen and the small intestine.

The effects of MCE (HG) on VFA concentrations and the molar percentages in the rumen are presented in Table 9. Supplementation of MCE did not affect (P > 0.100) the individual or total VFA concentrations which are determined primarily by the rate of carbohydrate fermentation and the rate of VFA absorption in the rumen. Considering the greater potential for VFA absorption indicated by longer papillae in HG, perhaps, MCE-fed calves produced more VFA than CTL to maintain similar concentrations between the two groups. However, molar percentages of VFA were also not statistically different, even though the percentage of propionate (P = 0.308) and butyrate (P = 0.232) in HG were >20% greater than CTL. On the other hand, Aguilar-Hernandez et al. (2016) report increased molar percentage of acetate and decreased molar percentages of valerate and isovalerate in the rumen of steers fed a different MCE preparation namely Sangrovit-RS

(Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany). Differences in concentrations of the active ingredients (e.g., sanguinarine) and other physiochemical properties among MCE preparations can result in different responses related to digestive parameters in ruminants.

Effects of MCE on microbiota composition in the small intestine

***Digesta-associated microbiota in the jejunum*:** Treatment effects on the alpha-diversity indices are given in Table 10. The number of species observed was similar between CTL and HG. Moreover, none of the alpha-diversity indices was significantly different between CTL and HG indicating that MCE supplement did not affect the richness and the distribution of the abundance taxonomic groups in the digesta. While alpha diversity describes the overall distribution of the abundance of taxonomic groups, the heat maps given in Figure 2 present the relative abundance of the 25 most abundant genera in jejunal digesta samples of CTL and HG. *Methanobrevibacter, Olsenella, Bifidobacterium, Lachnospiraceae_UC,* and *Coprococcus* were the most abundant genera in the present digesta samples. Malmuthuge et al. (2014), however, reported different genera (*Sharpea, Butyrivibrio, Ruminococcus, and Lactobacillus*) to be the most abundant in pre-weaned Holstein bull calves. Another study identified a further different set of genera including *Butyrivibrio, Lachnospiraceae_UC, Acetitomaculum*, *Peptostreptococcaceae_UC*, and *Clostridiales_UC* as the most abundant genera in the jejunal digesta of Holstein cows (Mao et al., 2015). Considering together, several factors including age, host genetics, and diet seem to considerably influence bacterial community composition in the small intestine of cattle. When analyzed with the top 100 OTUs, the relative abundance of five OTU was different ($P < 0.100$) between CTL and HG (Table 11). Representative sequences of each of those OTU were queried against the NCBI rRNA/ITS database by using NCBI BLAST to improve the taxonomic classification (Table 12). The relative abundance of *Methanobrevibacter* (OTU1) closely related to *Methanobrevibacter wolinii* (98.82% identity) tended to be greater in HG than CTL ($P = 0.077$).

On the other hand, *Methanobrevibacter* (OTU76) closely related to *Methanobrevibacter millerae* (98.82% identity) tended to be less abundant in HG than CTL. Both species are methanogenic *Archaea* (Miller and Lin Ex, 2002; Rea et al., 2007). They are, however, different in fermentation substrate requirements. *Methanobrevibacter wolinii* requires coenzyme M and branched-chain fatty acids (Miller and Lin, 2002), whereas *Methanobrevibacter millerae* does not have such requirements (Rea et al., 2007). We speculate a link between MCE and those requirements that would explain the opposite effect of MCE on those two species. *Bifidobacterium_UC* (OTU06) tended to have a lower relative abundance in HG compared to CTL (P = 0.085). We could not describe the role of *Bifidobacterium* (OTU06) in the small intestine as the sequence was closely related to multiple species in the NCBI BLAST search emphasizing that the 16S rRNA sequencing has an insufficient resolution to differentiate between some bacterial species. The relative abundance of *Eubacterium* (OTU23), closely related to *Eubacterium pyruvativarans* (100% identity), tended to be higher in HG than CTL (P = 0.071). This species is a non-saccharolytic bacteria relying on peptides and single amino acids as a source of both nitrogen (N) and energy (Wallace et al., 2004; Wallace et al., 2003 and Eschenlauer et al., 2002). A previous study demonstrated that an MCE supplementation increased the duodenal flows of N in cattle (Aguilar-Hernandez et al., 2016), indicating an increased availability of nutrients for non-saccharolytic bacteria in the small intestine. *Gastranaerophilales_ge* (OTU75) was another OTU that did not match well (<90% similarity) with any species in NCBI rRNA/ITS database (Table 12). Nonetheless, the abundance of *Gastranaerophilales_ge* (OTU75) in CTL was greater than HG (P = 0.045) and was positively related to DMI (r = 0.55, P = 0.018) and ADG (r = 0.61, P = 0.007) suggesting that MCE could negatively affect some beneficial bacteria in the small intestine.

***Mucosa-associated microbiota in the jejunum*:** Similar to our observations of the

microbiota in the digesta, the MCE supplementation did not affect the alpha diversity of microbiota associated with the mucosa of the jejunum. The relative abundance of the 25 most abundant genera in CTL and HG are presented in the heatmap of Figure 3. *Methanobrevibacter, Olsenella, Lachnospiraceae_UC, Bifidobacterium_UC,* and *Clostridia_UCG-014_ge* are the most abundant genera in the mucosa. The most abundant genera in pre-weaned calves reported by Mulmuthuge et al. (2014) are different from the present list supporting the idea that weaning is a critical factor shaping up gut microbiota composition (Meale et al., 2017). Table 13 gives the mean abundances of OTUs that responded ($P < 0.100$) to MCE. A greater number of OTU in the mucosa responded to MCE compared to those in the digesta. *Blautia* (OTU16) related to *Blautia wexlerae DSM 19850* (91.8% identity, Table 13) was less abundant in HG than CTL ($P = 0.020$). *Blautia* is a major genus in the lower gut of many mammals including humans and ruminants (Eren et al., 2015; Franks et al., 1998; Rieu-Lesme et al., 1996). The exact role of *Blautia wexlerae DSM 19850* is unclear but our correlation analysis revealed a negative relationship between the ADG: DMI and the abundance of *Blautia wexlerae DSM 19850* ($r = -0.54$, $P = 0.023$) suggesting MCE can improve feed efficiency of post-weaned calves by affecting mucosa-associated bacteria in the small intestine. *Megasphaera* (OTU51), closely related to *Megasphaera elsdenii* (98.8% identity) had a lower relative abundance in HG than CTL. *Megasphaera elsdenii* are Gram-negative bacteria capable of fermenting lactate and therefore, play a critical role in mitigating rumen acidosis in cattle (Hobson et al., 1958; Marounek et al., 1989; Chen, et al., 2019;). However, the importance of *Megasphaera elsdenii* in the lower gut of ruminants is poorly understood. Similar to the results of the digesta, the abundance of *Methanobrevibacter millerae* tended to be lower in HG compared to CTL ($P = 0.075$) and negatively related to ADG: DMI ($r = -0.43$, $P = 0.081$, Table 13) supporting the idea that MCE can modulate the mucosa-associated microbiota composition and promote feed

efficiency. Calves supplemented with MCE had a lower abundance (P = 0.018) of *Clostridioides* (OTU76) which was closely related to *Clostridioides difficile* (98.8% identity). This finding was very intriguing as *Clostridioides difficile* is one of the most studied human enteropathogens and is the leading cause of antibiotic-associated diarrhea worldwide. *Clostridioides difficile* infections can be zoonotic, and livestock can serve as the reservoirs (Weese, 2020). Rodriguez et al. (2017) isolated *Clostridioides difficile* from feces of non-diarrheic and clinically healthy calves and adult cattle, and all the isolates were toxic. Moreover, there was a higher probability of colonization of *Clostridioides difficile* in calves < 6 mon. of age than mature cattle (Bandeji et al., 2016; Rodriguez et al., 2017). Supporting our finding, Kudera et al. (2020) showed sanguinarine decreased the abundance of *Clostridioides difficile* in vitro. Moreover, *Clostridioides difficile* abundance was negatively correlated with feed conversion efficiency (r = -0.55, P = 0.020) in the present study. Considering together, feeding MCE to calves can increase feed efficiency and decrease the excretion of zoonotic pathogens in feces, both having important implications in the sustainability of livestock production.

Supplementation of MCE increased the relative abundance of OTU90 (P = 0.018) and OTU94 (P = 0.066), closely related to *Bifidobacterium faecale* (99.6% identity) and *Mogibacterium timidum* (94.86% identity), respectively. It should be acknowledged that *Mogibacterium timidum* was previously referred to as *Eubacterium timidum* (Holdeman et al., 1980; Nakazawa et al., 2000). Nonetheless, little information is available about the functions of those species which are Gram-positive, non-motile, and strictly anaerobic. (Choi et al., 2014). The genus *Bifidobacterium* is, however, known to improve the overall health of the host animal (Rivière et al., 2016). Moreover, Gardiner et al. (2020) reviewed several studies focused on intestinal microbiota in pigs and highlighted a negative relationship between the abundance of

genus *Mogibacterium* and feed efficiency. Our correlation analysis did not reveal any associations of *Mogibacterium timidum* with ADG: DMI in calves (Table 13). The abundance of OTU96 related to *Anaerocolumna jejuensis* (90.2% identity) was greater in HG than CTL (P = 0.010). To our knowledge, this is the first time *Anaerocolumna jejuensis* is reported to be found in the intestinal digesta of calves. A little information is available in the literature and our correlation analysis did not provide any evidence to describe the implications of having a high abundance of *Anaerocolumna jejuensis* in the gut. The abundance of OTU98, tended to be lower in HG than CTL (P = 0.092). Because there were no species closely related to this OTU in our NCBI BLAST search, we were unable to determine the potential functions.

Effects of MCE on microbiota composition in rumen liquid and solids

***Solid-associated microbiota*:** Treatment effects on the alpha diversity, including the total number of bacterial OTUs observed, Chao1, Shannon, Simpson, and Inverse Simpson are provided in Table 10. None of the alpha-diversity indices was significantly different between CTL and HG. The relative abundance of the 25 most abundant genera in all mucosa samples in CTL and HG are presented in the heatmap of Figure 4. The five most abundant genera included *Lachnospiraceae_UC, Prevotella, Clostridia_UCG-014_ge, Lachnospiraceae_NK3A20_group,* and *Erysipelotrichaceae_UCG-002.* Consistent with the results of jejunal content, *Methanobrevibacter* (OTU7), closely related to species *Methanobrevibacter wolinii* (98.82% identity) tended to have higher relative abundance in HG than CTL (P = 0.080). Additionally, OTU46, closely related to species *Methanobrevibacter boviskoreani* tended to have a lower relative abundance in HG compared to CTL. We also found that *Methanobrevibacter boviskoreani* was positively correlated with ADG and DMI but did not have a relationship with ADG: DMI (Table 14). Generally, methane production in the rumen is associated with decreased feed efficiency (Tapio et al., 2017). The abundance of methanogens, however, can increase when feeds

are highly digestible in the rumen. The digestibility of feeds can be positively correlated with DMI and ADG. Nonetheless, the present relationships of microbiota abundance with ADG must be evaluated carefully as a common ADG was calculated for whole post-weaning period, whereas the microbiota abundance was assessed with samples collected only on the last day of that period. Similar to our observation in the jejunal mucosa, OTU60, closely related to the species *Blautia caecimuris* tended to be lower in HG compared to CTL.

***Liquid-associated microbiota*:** The relative abundance of the 25 most abundant genera in CTL and HG are presented in the heatmap of Figure 5. The five most abundant genera included *Lachnospiraceae_UC*, *Clostridia_UCG-014_ge*, *Olsenella*, *Lachnospiraceae_NK3A20_group*, and *Erysipelotrichaceae_UCG-002*. The abundance of OTU02, closely related to the species *Olsenella umbonata* tended to be higher in HG than CTL ($P = 0.080$, Table 14). The members of the genus *Olsenella* are Gram-positive and non-motile bacteria that ferment carbohydrates predominantly to lactic acid. They were first isolated from the ruminal fluid of the sheep (Kraatz et al., 2011). OTU29, closely related to *Kineotrix alysoides*, had a lower relative abundance in HG when compared to CTL. To our knowledge, there is not much information about the potential role of *Kineotrix alysoides* in the rumen. It is, however, identified as butyrate-producing anaerobic bacteria (Haas et al., 2017). The OTU89 closely related to *Blautia caecimuris* was less abundant in HG than CTL (Table 14). This observation, once again, in line with the effects of MCE on the abundance of *Blautia caecimuris* in jejunal mucosa and rumen solids. Those consistent results emphasize the robustness of the effects of MCE on gut microbiota composition in calves. Moreover, the consistency of the results can also be a part of the sampling and DNA extraction procedures in the present study being consistent and acceptable.

Conclusions

In the current study, dietary supplementations of graded levels of a commercial preparation of MCE increased milk replacer intake and tended to increase DMI of pre-weaned calves specifically for the highest dose. Agreeing with the fact that nutrient intake is a major drive to gut development in young calves, the highest dose was related to longer papillae in the rumen, and increased villus height and VCR in the small intestine. The supplementation of MCE did not change the VFA concentrations in the rumen, and alpha diversity of microbiota communities in the rumen and the small intestine. However, it affected the abundance of some individual species in both compartments. For instance, the relative abundance of *Clostridioides difficile*, a zoonotic pathogen, was lower in jejunal digesta of MCE-supplemented calves compared to control calves. The abundance of some species affected by MCE was related to ADG or feed conversion efficiency across the treatments. However, none of the MCE doses affected ADG or feed efficiency in the present study. The majority of the parameters in blood chemistry and hematology analyses did not change with the MCE dose and the values of all the parameters were within the reference ranges of healthy calves. Therefore, the dietary supplementations of MCE tested in the present study seem to be safe for calves. More robust conclusions about the safety of the supplements could be drawn once the results of MCE residue concentrations in tissues (e.g., muscle, liver, and fat) are available.

CHAPTER 5. GENERAL CONCLUSIONS

This thesis covers an experiment where effects of a phytogenic feed additive, claimed to possess anti-inflammatory, antioxidant, and antimicrobial properties, on dry matter intake (DMI), the growth, biomarkers of the health and safety, and important aspects of gastrointestinal tract development in calves were investigated. In recent years, feed additives with such properties are garnered significant attention as a result of growing concerns among the general public as well as scientific communities about improving the welfare of farm animals while mitigating potential contribution of animal farms to antibiotic resistance. In developing alternatives for antibiotics, medicinal plants such as *Macleaya cordata* have been thoroughly studied for antimicrobial properties of secondary metabolites they produce. The company Phytobiotics (Eltville, Germany) is a world leader in manufacturing feed additives based on *Macleaya cordata* plant extracts (MCE). Those MCE have been shown to improve feed intake, growth, and feed efficiency primarily by modulating gut morphology, microbiota composition in the gut, and immune function in monogastric animals such as pigs and poultry. Because of the complexity of the gastrointestinal tract of ruminant animals, the findings of monogastric species cannot be directly extrapolated and thus new studies are required to explore the roles of MCE in ruminants.

Dietary MCE increased milk replacer consumption of pre-weaned calves in the present study. Nonetheless, the MCE supplements decreased starter intake in both pre-weaned and weaned calves. The lower starter intake pre-weaning can be partly a result of elevated nutrient intake via milk replacer (Khan, et al., 2011). The lower starter intake for MCE supplementation post-weaning, however, suggests a negative impact on MCE on appetite and other mechanisms regulating the voluntary feed intake or improved dietary nutrient utilization in the body for MCE in the diet. The uncompromised average daily gain (ADG) of MCE-supplemented calves post-

weaning supports the latter. Furthermore, the MCE supplementation increased the length of papillae in the rumen as well as villus height in the small intestine corroborating the potential for an improved nutrient availability for bodily functions including growth. Nonetheless, in the present study, ADG of pre- and post-weaning periods were determined with BW measurements recorded just at the beginning and the end of each period. More frequent body weight measurements could have helped better describe the associations between MCE supplements and ADG. Moreover, a greater sample size would have also helped capture the true effect of MCE on growth. It is fair to mention that the sample size of the present study was determined for the variables indicating the safety of the MCE doses, which is a critical component of evaluating phytogenic feed additives for a given species.

In the current study, we evaluated the safety of MCE by using blood chemistry and hematology with respect to four doses in the diet. We also collected tissues including skeletal muscle, liver, kidney, and fat for the analyses of MCE residue concentrations in those tissues as a part of the safety assessment. We could not, however, report those concentrations in this thesis due to a delay in the analytical laboratory end. The doses of MCE (2, 5, 10 g/d pre-weaning and 4, 10, 20 g/d post-weaning) seem to be safe as all blood chemistry and hematology parameters were within the reference ranges of healthy calves (Panousis et al. 2018; Klinkon and Ježek, 2012). Nonetheless, assessing the tissue residue concentrations and changes in markers specific to DNA damage (Kudera et al., 2020; Ansari et al., 2005) would allow for more robust conclusions about the safety of the MCE doses.

Because gut microbiota plays a key role in growth and development of young calves and MCE is known to possess antibacterial properties, evaluating the impact of feeding MCE not only on the host animal but also the microbiota inhabiting the gut is well justified. In this regard, MCE

fed at a high dose (10g and 20g/animal/d during pre- and post-weaning, respectively) did not affect the volatile fatty acid profiles in the rumen or the number of observed bacterial species and the distribution of their abundance in the rumen as well as in the small intestine. It is noteworthy that those results were from a single sampling point of calves at 95 d of age. Because gut development responds significantly to several environmental factors in young calves (Jami et al., 2013 and Rey et al., 2014), evaluating VFA profiles, gut morphology, and gut microbiota composition with periodically collected samples would have helped better describe the effects of MCE.

Despite the absence of an effect on the overall diversity of gut microbiota, the MCE supplementation affected the abundance of some individual bacteria species in both the rumen and the small intestine. In jejunal digesta, the relative abundance of *Gastranaerophilales_UC* having positive relationships with ADG and DMI decreased for the MCE supplementation. On the other hand, in jejunal mucosa, the abundance of *Blautia wexlerae DSM 19850* and *Methanobrevibacter millerae*, each having a negative association with feed efficiency (ADG: DMI) decreased for the MCE supplementation. The present microbiota analysis did not, however, suggest an underlying mechanism for improved gut morphology in MCE-supplemented calves as none of the bacteria responding significantly to dietary MCE were related to papillae length in the rumen or villus height in the small intestine. On the other hand, feeding MCE significantly decreased the abundance of *Clostridioides difficile* in jejunal digesta. Calves are considered significant reservoirs of this zoonotic pathogen. Therefore, MCE in the diet can have a positive impact on the sustainability of dairy or beef production systems by mitigating the pathogen load in feces of calves.

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APPENDIX. TABLES AND FIGURES.

Table 1. Commercial preparations of *Macleaya cordata* extract (MCE)and corresponding doses fed during pre- and post-weaning.

Treatment	Sangrovit Stat Pak® (Pre-weaning, 5 to 49 d)	SangrovitG premix® (Post-weaning, 50 to 95 d)
CTL (Control)	0 g/animal/day	0 g/animal/day
LW (Low)	2 g/animal/day	4 g/animal/day
MD (Medium)	5 g/animal/day	10 g/animal/day
HG (High)	10 g/animal/day	20 g/animal/day

Table 2. Ingredient and nutrient composition of milk replacer and starter ration.

Item	Content
----------Milk Replacer----------	
Nutrient composition (% of DM unless otherwise specified)[1]	
Crude protein, min	22.00
Crude fat, min	20.00
Crude fiber, max	0.15
Calcium, min	0.75
Calcium, max	1.25
Phosphorus, min	0.70
Vitamin A, min	9072 IU/kg
Vitamin D3, min	2268 IU/kg
Vitamin E, min	45 IU/kg
----------Starter ration----------	
Ingredient composition (% of as-fed)[1]	
Whole corn, shelled	54.0
Whole oats	7.5
Non-medicated heifer pellet	35.0
Molasses, liquid	2.5
Corn oil	1.0
Nutrient composition (% of DM, unless otherwise specified)[2]	
Dry matter, %	87.9
Crude protein	20.2
Neutral detergent fiber	15.2
Acid detergent fiber	7.3
Starch	41.5
Non-structural carbohydrates	54.0
Crude fat	4.2
Ash	6.3

[1]Reported by the manufacturer; [2]Analyzed in a certified laboratory

Table 3. Effects of MCE dose on feed intake, average daily gain (ADG), and feed efficiency (ADG: DMI) during pre- and post- weaning.

Response variable	Least squares means[1]				SEM	*P*-value	*P*-value		
	CTL	LW	MD	HG			Linear	Quadratic	Cubic
Pre-weaning (5-49 d of age)									
MR intake, kg/d	4.91	5.22	5.09	5.12	0.05	<0.001	0.054	0.014	0.001
Starter intake, kg/d	0.65	0.53	0.67	0.67	0.02	<0.001	0.005	0.214	<0.001
DMI[2], kg/d	1.17	1.1	1.21	1.21	0.02	<0.001	0.001	0.676	<0.001
ADG, kg/d	0.75	0.68	0.7	0.76	0.06	0.719	0.668	0.423	0.651
ADG:DMI	0.62	0.64	0.61	0.64	0.02	0.684	0.677	0.614	0.351
BW[4], kg	79.65	76.57	77.64	79.98	2.97	0.886	0.721	0.438	0.639
Post-weaning (50-95 d of age)									
Starter intake, kg/d	3.56	3.36	3.5	3.52	0.05	0.068	0.571	0.148	0.013
DMI[3], kg/d	3.17	2.99	3.12	3.14	0.04	0.082	0.569	0.142	0.010
ADG, kg/d	1.47	1.44	1.47	1.40	0.11	0.959	0.680	0.888	0.753
ADG:DMI	0.45	0.46	0.46	0.45	0.02	0.962	0.932	0.883	0.831
BW[5], kg	146.06	141.61	145.36	138.86	7.66	0.815	0.572	0.859	0.608

[1]different superscripts indicate significantly different least squares means ($P < 0.05$) of control (CTL), and low (LW), medium (MD), and high (HG) doses of MCE

[2]DMI = (Milk replacer intake × 0.12) + (Starter intake × 0.88)

[3]DMI = (Starter intake × 0.88)

[4]body weight at 49 d of age (45 d after feeding MCE)

[5]body weight at 95 d of age (90 d after feeding MCE)

Table 4. Effects of MCE dose on blood chemistry pre-weaning (49 d of age).

Parameter	Pre-weaning				*P*-value		
	CTL	LW	MD	HG	Linear	Quadratic	Cubic
Albumin, g/dL	3.27	3.39	3.33	3.44	0.176	0.737	0.529
Total protein, g/dL	5.82	6.05	6.05	5.92	0.944	0.426	0.929
Blood urea nitrogen, mg/dL	9.86	11.18	10.63	9.98	0.606	0.334	0.176
Alkaline phosphatase, U/L	233.72	227.38	214.94	239.96	0.961	0.800	0.392
Alanine aminotransferase, U/L	11.37	11.74	12.05	12.16	0.664	0.758	0.750
Asparate aminotransferase, U/L	60.13	55.05	53.42	56.82	0.558	0.541	0.968
Lactate dehydrogenase, U/L	740.05	721.16	749.49	731.8	0.927	0.959	0.583
γ-Glutamyltransferase, U/L	18.34	19.53	17.41	15.87	0.087	0.736	0.645
Creatine kinase, U/L	102.68	85.3	99.46	92.35	0.282	0.985	0.230
Creatinine, mg/dL	0.84	0.96	0.87	0.95	0.058	0.652	0.042
Total bilirubin, mg/dL	0.17	0.16	0.11	0.13	0.185	0.800	0.047
Cholesterol, mg/dL	73.66	86.7	87.77	85.27	0.150	0.087	0.421
Glucose, mg/dL	89.31	90.89	93.37	94.87	0.203	0.866	0.623
Calcium, mg/dL	9.64	9.67	9.82	9.85	0.178	0.835	0.066
Inorganic phosphorus, mg/dL	8.95	9.48	9.42	9.35	0.161	0.327	0.075
Sodium, mEq/L	139.98	140.97	140.41	141.61	0.059	0.802	0.820
Potassium, mEq/L	4.88	4.94	4.8	4.68	0.151	0.913	0.863
Chloride, mEq/L	101.67	101.42	101.48	100.96	0.540	0.925	0.289
Magnesium, mg/dL	2.23	2.24	2.22	2.28	0.651	0.913	0.619

Table 5. Effects of MCE dose on blood chemistry post-weaning (95 d of age).

Parameter	Least squares means				*P*-value		
	CTL	LW	MD	HG	Linear	Quadratic	Cubic
Albumin, g/dL	3.64	3.79	3.77	3.77	0.203	0.273	0.481
Total protein, g/dL	5.94	5.98	6.06	5.82	0.595	0.140	0.737
Blood urea nitrogen, mg/dL	11.36	12.87	13.23	12.38	0.709	0.421	0.480
Alkaline phosphatase, U/L	237.78	227.07	235.84	269.36	0.736	0.930	0.537
Alanine aminotransferase, U/L	15.07	15.24	14.95	15.36	0.889	0.637	0.735
Asparate aminotransferase, U/L	74.31	69.65	73.97	79.54	0.181	0.876	0.034
Lactate dehydrogenase, U/L	837.45	816.16	870.61	846.85	0.423	0.143	0.004
γ-Glutamyltransferase, U/L	18.34	19.53	17.41	15.87	0.101	0.814	0.424
Creatine kinase, U/L	138.32	131.42	121.39	136.66	0.649	0.248	0.261
Creatinine, mg/dL	10.04	8.53	9.01	5.77	0.499	0.657	0.620
Total bilirubin, mg/dL	0.13	0.17	0.14	0.14	0.960	0.916	0.812
Cholesterol, mg/dL	62.16	73.00	69.57	58.47	0.357	0.211	0.391
Glucose, mg/dL	107.01	103.69	101.87	104.62	0.114	0.163	0.984
Calcium, mg/dL	10.14	10.27	10.20	10.18	0.721	0.907	0.963
Inorganic phosphorus, mg/dL	9.64	9.66	9.56	9.65	0.941	0.925	0.388
Sodium, mEq/L	139.08	140.27	139.71	139.51	0.792	0.783	0.688
Potassium, mEq/L	4.80	4.75	4.74	4.47	0.769	0.923	0.150
Chloride, mEq/L	97.37	98.12	98.58	99.56	0.003	0.899	0.445
Magnesium, mg/dL	2.21	2.22	2.19	2.29	0.300	0.402	0.152

Table 6. Effects of MCE dose on hematology parameters pre-weaning (49 d of age).

Variable	Least squares means				*P*-value		
	CTL	LW	MD	HG	Linear	Quadratic	Cubic
White blood count, x10³/µL	8.84	8.16	8.43	8.11	0.465	0.374	0.132
Red blood count, x10⁶/µL	10.04	10.35	10.45	10.42	0.430	0.727	0.844
Hemoglobin concentration, g/dL	11.80	12.46	12.11	12.20	0.435	0.954	0.689
Hematocrit, %	34.34	36.62	35.65	35.73	0.493	0.778	0.525
Mean corpuscular volume, fL	34.26	35.46	34.12	34.35	0.761	0.828	0.289
Mean corpuscular hemoglobin, pg	11.76	12.09	11.59	11.71	0.799	0.734	0.366
Mean corpuscular hemoglobin, g/dL	34.36	34.07	34.00	34.15	0.740	0.602	0.439
Red cell distribution width, %	19.01	18.94	19.54	18.74	0.690	0.078	0.048
Platelet count, x10³/µL	681	522	547	584	0.531	0.234	0.404
MPV, fL	7.40	7.11	7.41	7.74	0.159	0.130	0.743
Neutrophils, %	31.79	31.22	32.85	31.72	0.963	0.818	0.842
Lymphocytes, %	57.24	58.81	56.38	59.14	0.820	0.750	0.568
Monocytes, %	8.49	7.61	7.73	6.88	0.435	0.875	0.421
Eosinophils, %	1.63	1.60	2.05	1.81	0.377	0.311	0.192
Basophils, %	1.14	1.11	1.08	0.95	0.246	0.762	0.816
Absolute Neutrophils, x10³/µL	2.80	2.64	2.80	2.63	0.837	0.682	0.252
Absolute Lymphocytes, x10³/µL	5.07	4.69	4.73	4.74	0.510	0.358	0.355
Absolute Monocytes, x10³/µL	0.74	0.63	0.64	0.56	0.311	0.691	0.140
Absolute Eosinophils, x10³/µL	0.14	0.14	0.17	0.14	0.405	0.405	0.084
Absolute Basophils, x10³/µL	0.10	0.09	0.09	0.08	0.172	0.681	0.287

Table 7. Effects of MCE dose on hematology parameters post-weaning (95 d of age).

Variable	Least squares means				*P*-value		
	CTL	LW	MD	HG	Linear	Quadratic	Cubic
White blood count, $x10^3/\mu L$	9.21	9.77	9.31	8.15	0.109	0.420	0.690
Red blood count, $x10^6/\mu L$	10.04	9.79	9.87	10.22	0.533	0.385	0.542
Hemoglobin concentration, g/dL	12.85	13.22	12.91	13.40	0.194	0.881	0.140
Hematocrit, %	36.11	36.86	35.79	37.72	0.234	0.395	0.116
Mean corpuscular volume, fL	35.89	37.71	36.41	36.97	0.529	0.542	0.217
Mean corpuscular hemoglobin, pg	12.77	13.50	13.14	13.15	0.554	0.152	0.491
Mean corpuscular hemoglobin, g/dL	35.64	35.81	36.02	35.54	0.932	0.305	0.387
Red cell distribution width, %	22.17	21.68	23.14	22.34	0.513	0.395	0.085
Platelet count, $x10^3/\mu L$	614	546	586	513	0.349	0.947	0.524
Mean Platelet Volume, fL	6.87	9.13	9.08	7.83	0.799	0.063	0.517
Neutrophils, %	30.24	37.73	35.93	33.96	0.805	0.105	0.097
Lymphocytes, %	59.62	53.16	54.47	58.18	0.703	0.079	0.204
Monocytes, %	6.79	5.97	5.69	4.89	0.150	0.966	0.587
Eosinophils, %	1.89	2.09	2.47	1.57	0.207	0.835	0.210
Basophils, %	1.73	1.47	1.74	1.84	0.432	0.802	0.256
Absolute Neutrophils, $x10^3/\mu L$	2.78	3.78	3.35	2.79	0.398	0.160	0.144
Absolute Lymphocytes, $x10^3/\mu L$	5.48	5.13	5.07	4.73	0.100	0.773	0.495
Absolute Monocytes, $x10^3/\mu L$	0.65	0.56	0.55	0.41	0.047	0.874	0.378
Absolute Eosinophils, $x10^3/\mu L$	0.17	0.19	0.20	0.12	0.080	0.708	0.237
Absolute Basophils, $x10^3/\mu L$	0.16	0.14	0.16	0.15	0.627	0.754	0.491

Table 8. The least square means and associated standard error of the mean (SEM, n = 10) for parameters representing gut morphology in control (CTL) and MCE-supplemented calves (HG).

Variable	CTL	HG	SEM	*P*-value
Rumen				
Papillae length, mm	2.68	3.45	0.16	0.013
Papillae width, mm	0.51	0.51	0.01	0.871
Duodenum				
Villus height, mm	0.45	0.57	0.03	0.01
Crypt depth, mm	0.48	0.47	0.03	0.614
Villus height: crypt depth	1.02	1.4	0.11	0.027
Jejunum				
Villus height, mm	0.48	0.52	0.03	0.128
Crypt depth, mm	0.55	0.47	0.03	0.033
Villus height: crypt depth	0.95	1.34	0.11	0.022
Ileum				
Villus height, mm	0.52	0.62	0.03	0.028
Crypt depth, mm	0.48	0.47	0.03	0.566
Villus height: crypt depth	1.19	1.34	0.11	0.065

Table 9. The least square means and associated standard error of the mean (SEM, n = 10) for volatile fatty acid (VFA) concentrations and molar percentages of VFA in the rumen of control (CTL) and MCE-supplemented calves (HG).

Variable	CTL	HG	SEM	*P*-value
VFA Concentration, mM				
Acetate	81.50	75.50	7.60	0.541
Propionate	32.08	31.62	3.40	0.918
Butyrate	12.49	11.77	2.49	0.823
Valerate	3.97	3.92	0.50	0.940
Isovalerate	7.53	4.15	1.72	0.147
Isobutyrate	0.84	0.89	0.06	0.490
Total VFA	138.43	127.82	9.00	0.374
VFA molar %				
Acetate	59.68	59.73	3.06	0.990
Propionate	23.31	28.25	3.66	0.308
Butyrate	9.01	14.68	3.56	0.232
Valerate	3.70	4.60	1.25	0.580
Isovalerate	7.61	5.45	3.14	0.600
Isobutyrate	0.83	1.12	0.37	0.551
Propionate: acetate	0.40	0.49	0.07	0.323

Table 10. Alpha diversity indices in control (CTL) and calves fed MCE at the highest dose (HG) at 95 d of age for rumen solid, rumen fluid, jejunal content and jejunal mucosa and their corresponding *P*-values (n = 10).

Index	CTL	HG	SEM	*P*-value	CTL	HG	SEM	*P*-value
Jejunal Microbiota								
	Digesta-associated				Mucosa-associated			
# of species	514	535	41.1	0.718	366	294	42.2	0.240
Chao1	629	669	59.8	0.616	482	383	59.6	0.255
Shannon	3.49	3.35	0.16	0.536	3.88	3.51	0.25	0.301
Simpson	0.92	0.92	0.01	0.975	0.93	0.92	0.03	0.801
Inverse-simpson	15.4	14.0	2.69	0.685	21.4	15.9	5.35	0.470
Rumen Microbiota								
	Solid-associated				Liquid-associated			
# of species	476	475	43.3	0.991	363	318	35.91	0.344
Chao1	633	636	64.1	0.964	552	458	60.6	0.238
Shannon	3.73	3.96	0.14	0.217	3.7	3.74	0.13	0.799
Simpson	0.94	0.96	0.01	0.204	0.93	0.94	0.01	0.273
Inverse-simpson	20.40	24.30	3.28	0.369	16.40	18.70	2.20	0.441

Table 11. The least squares means of the relative abundance of operational taxonomic units (OTU) in jejunal content that were considerably different ($P < 0.10$) between control and MCE-supplemented (HG) calves (n = 10) and their relationships with DMI, average daily gain (ADG), villus height, crypt depth, and villus height: crypt depth ratio (VCR).

OTU	Relative abundance		*P*-value	Correlations[1]					
	CTL	HG		DMI	ADG	ADG: DMI	Villus height	Crypt depth	VCR
Methanobrevibacter wolinii (OTU01)	0.061	0.149	0.077	-0.20	-0.31	-0.11	-0.01	-0.14	0.10
Bifidobacterium (OTU06)	0.036	<0.001	0.085	-0.20	-0.10	0.31	-0.33	0.38	-0.38
Eubacterium pyruvativorans (OTU23)	0.006	0.015	0.071	0.23	0.38	0.13	0.27	-0.21	0.23
Gastranaerophilales_UC (OTU75)	0.003	<0.001	0.045	0.55*	0.61*	-0.04	-0.26	0.23	-0.32
Methanobrevibacter millerae (OTU76)	0.008	<0.001	0.095	0.08	-0.10	-0.35	-0.22	0.23	-0.32

[1]Pearson's correlation coefficients.
*$P<0.05$

Table 12. NCBI taxonomy with correspondent coverage and identity for Operational Taxonomic Units (OTUs) significantly affected by treatment in jejunal content (JC), jejunal mucosa (JM), rumen liquid (RL), and rumen solid (RS).

Region	OTU	NCBI taxonomy	Coverage	Identity
JC	OTU00001	Methanobrevibacter wolinii	100%	98.82%
JC	OTU00006	Bifidobacterium*	100%	100%
JC	OTU00023	Eubacterium pyruvativorans	100%	100%
JC	OTU00075	No sequence available with > 90% identity		
JC	OTU00076	Methanobrevibacter millerae	100%	98.82%
JM	OTU00016	Blautia wexlerae DSM 19850	100%	91.83%
JM	OTU00050	No sequence available with > 90% identity		
JM	OTU00051	Megasphaera elsdenii	100%	100%
JM	OTU00058	Methanobrevibacter millerae	98%	99.20%
JM	OTU00076	Clostridioides difficile ATCC 9689 = DSM 1296	100%	98.81%
JM	OTU00090	Bifidobacterium faecale	100%	99.60%
JM	OTU00094	Mogibacterium timidum	100%	94.86%
JM	OTU00096	Anaerocolumna jejuensis	100%	90.20%
JM	OTU00098	Hallella seregens ATCC 51272	100%	91.70%
RL	OTU00002	Olsenella umbonata	100%	100%
RL	OTU00029	Kineothrix alysoides	100%	92.52%
RL	OTU00089	Blautia caecimuris	99%	90.51%
RS	OTU00007	Methanobrevibacter wolinii	100%	98.82%
RS	OTU00046	Methanobrevibacter boviskoreani JH1	100%	100%
RS	OTU00060	Blautia caecimuris	100%	90.16%

* Multiple species found with 100% coverage and 100% identity.

Table 13. The least squares means of the relative abundance of operational taxonomic units (OTU) in jejunal mucosa that were considerably different ($P < 0.10$) between control and MCE-supplemented (HG) calves (n = 10) and their relationships with DMI, average daily gain (ADG), villus height, crypt depth, and villus height: crypt depth ratio (VCR).

OTU	Relative abundance		*P*-value	Correlations[1]					
	CTL	HG		DMI	ADG	ADG: DMI	Villus height	Crypt depth	VCR
Blautia wexlerae DSM 19850 (OTU16)	0.0348	0.0063	0.020	0.22	<0.01	-0.55*	-0.11	-0.10	0.02
RF39_ge (OTU50)	0.0096	0.0037	0.062	-0.06	-0.24	-0.40	-0.13	-0.04	0.01
Megasphaera elsdenii (OTU51)	0.0051	0.0015	0.045	0.16	0.11	-0.13	-0.13	-0.07	-0.07
Methanobrevibacter millerae (OTU58)	0.0098	<0.0001	0.075	0.06	-0.13	-0.43**	-0.19	0.08	-0.18
Clostridioides difficile (OTU76)	0.0051	0.0012	0.018	-0.03	0.15	-0.55*	-0.13	0.04	-0.15
Bifidobacterium faecale (OTU90)	<0.0001	0.0038	0.018	0.12	0.08	-0.16	0.12	-0.06	0.04
Mogibacterium timidum (OTU94)	0.0004	0.0035	0.066	0.25	0.14	-0.34	0.12	-0.31	0.24
Anaerocolumna jejuensis (OTU96)	<0.0001	0.0033	0.010	0.06	0.06	-0.04	-0.17	-0.17	-0.07
Hallella seregens (OTU98)	0.0042	0.0001	0.092	0.10	-0.04	-0.28	-0.10	0.06	-0.14

[1]Pearson's correlation coefficients.
*P<0.05; **P<0.10

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Table 14. The least squares means of the relative abundance of operational taxonomic units (OTU) in rumen solids and liquid that were considerably different ($P < 0.10$) between control and MCE-supplemented (HG) calves (n = 10) and their relationships with DMI, average daily gain (ADG), villus height, crypt depth, and villus height: crypt depth ratio (VCR).

OTU	Relative abundance		*P*-value	Correlations[1]				
	CTL	HG		DMI	ADG	ADG: DMI	Papillae length	Papillae width
Rumen Liquid								
Olsenella umbonata (OTU02)	0.0025	0.0844	0.066	0.06	0.01	-0.11	0.10	0.06
Kineothrix alysoides (OTU29)	0.0151	0.0035	0.076	0.08	-0.10	-0.30	-0.07	0.25
Blautia caecimuris (OTU89)	0.0051	0.0002	0.038	-0.20	-0.30	-0.23	-0.45**	0.25
Rumen Solid								
Methanobrevibacter wolinii (OTU07)	0.0238	0.0508	0.080	-0.51	-0.45	0.30	-0.44	0.39
Methanobrevibacter boviskoreani (OTU46)	0.0079	<0.0001	0.068	0.44	0.69*	0.23	0.29	-0.38
Blautia caecimuris (OTU60)	0.0070	<0.0001	0.008	-0.33	-0.40	0.10	-0.20	0.78

[1]Pearson's correlation coefficients.

*P<0.05; **P<0.10

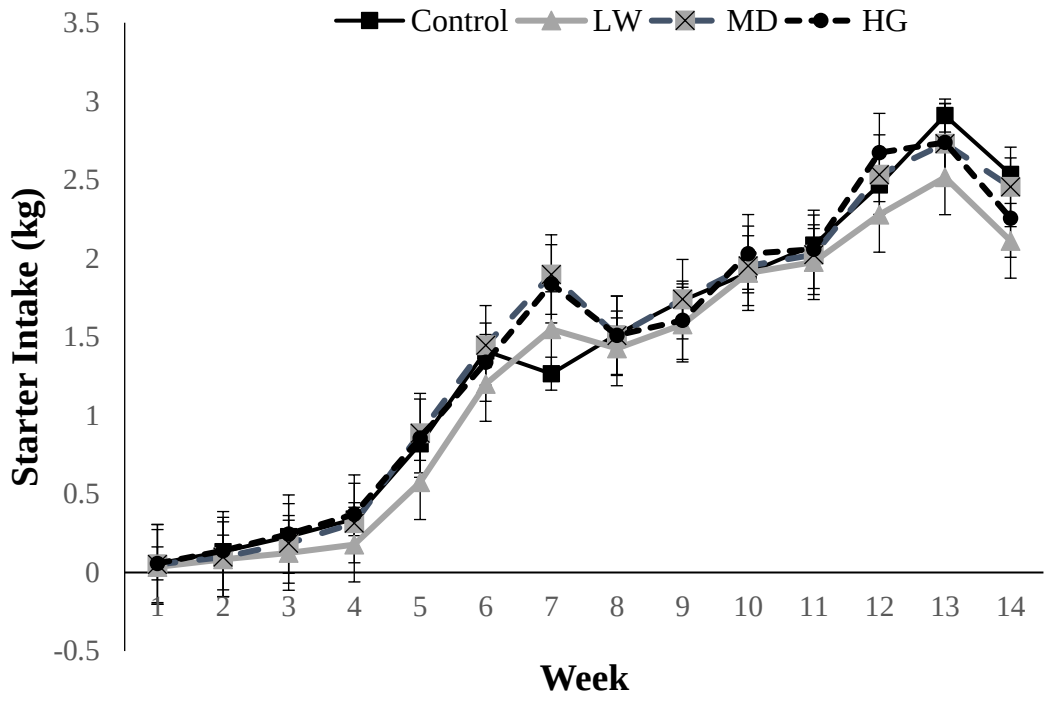

Figure 1. Average daily starter intake (kg) for control, low (LW), medium (MD), and high (HG) doses of MCE supplementation during weeks 1-13.

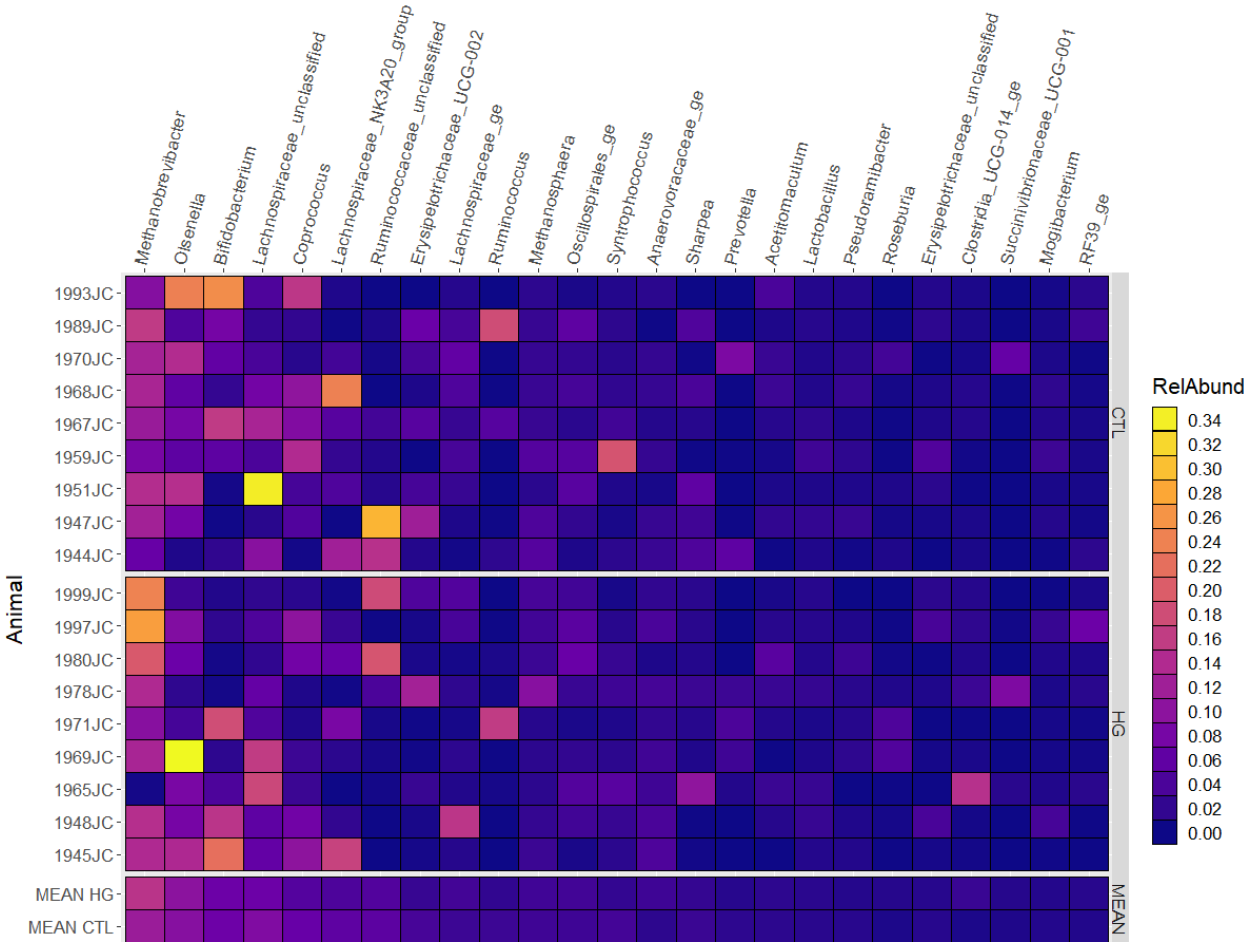

Figure 2. The relative abundance of the 25 most abundant genera in individual jejunal digesta samples and corresponding mean relative abundance of calves in control group (CTL) and calves receiving a high dose of MCE (HG).

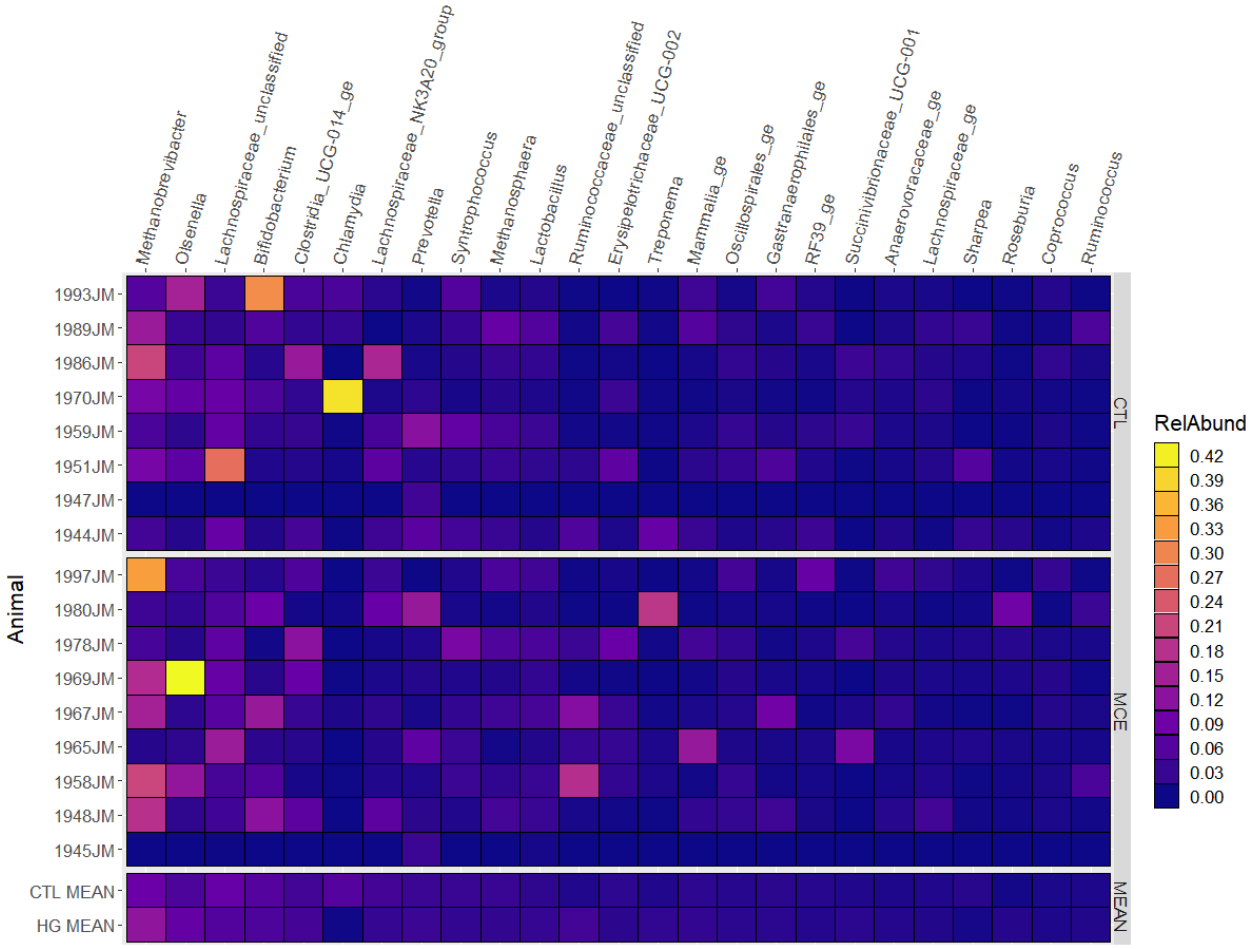

Figure 3. The relative abundance of the 25 most abundant genera in individual jejunal mucosa samples and corresponding mean relative abundance of calves in control group (CTL) and calves receiving a high dose of MCE (HG).

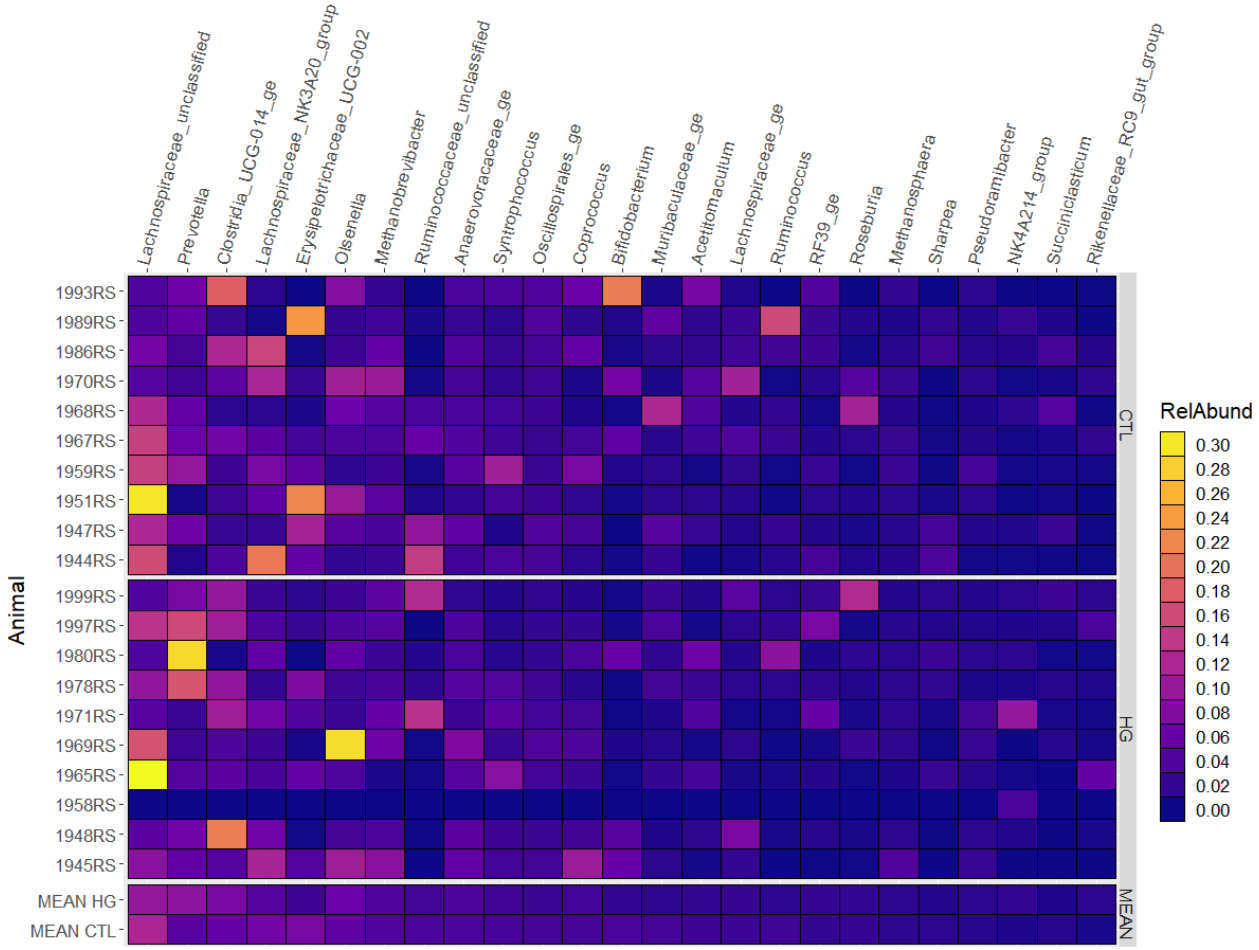

Figure 4. The relative abundance of the 25 most abundant genera in solid fraction of rumen digesta samples and corresponding mean relative abundance of calves in control group (CTL) and calves receiving a high dose of MCE (HG).

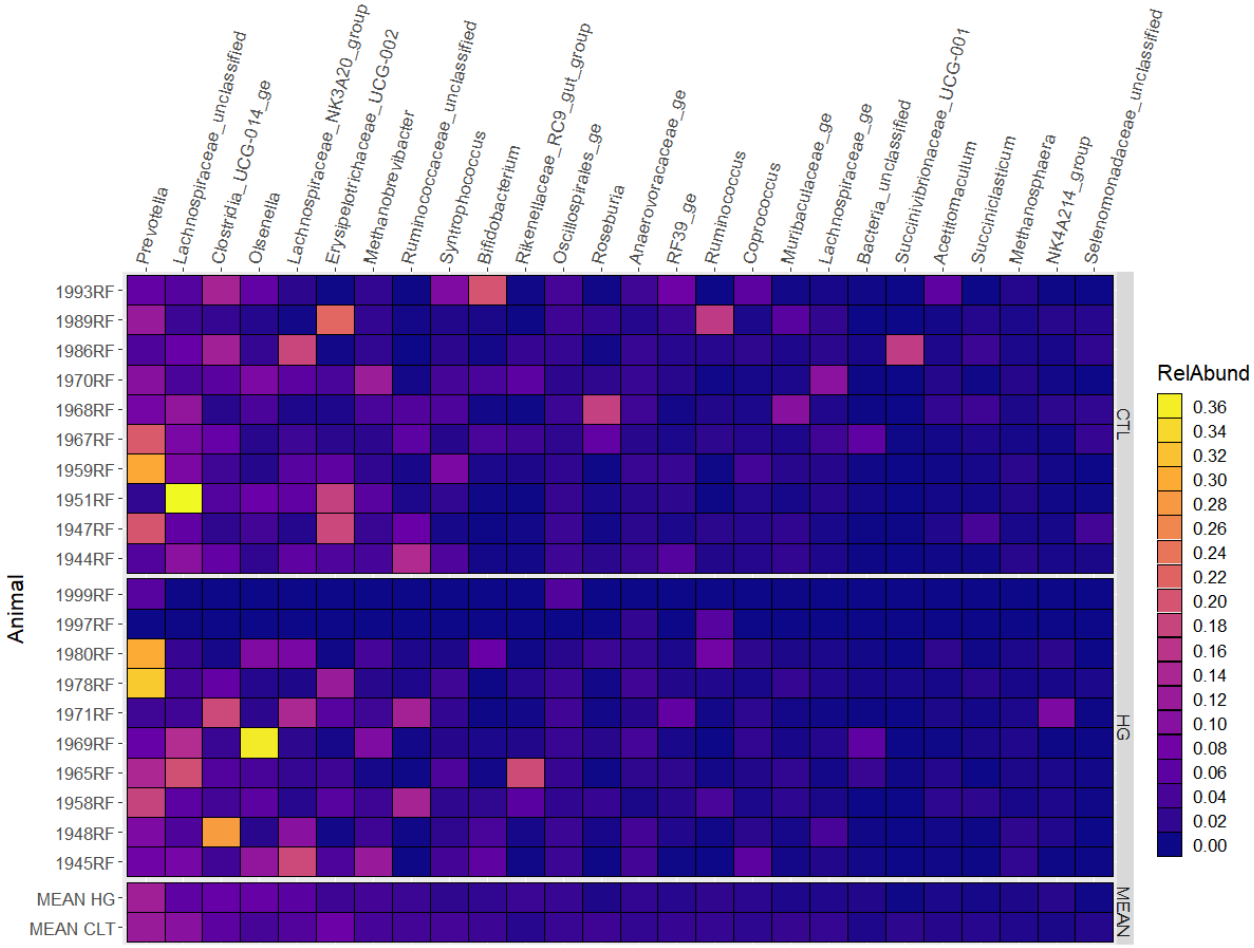

Figure 5. The relative abundance of the 25 most abundant genera in liquid fraction of rumen digesta samples and corresponding mean relative abundance of calves in control group (CTL) and calves receiving a high dose of MCE (HG).