Supplementing Sodium Butyrate to Limit-Fed Heifers: Effects on Growth, Health, and Nutrient Digestibility & Effect of Sodium Butyrate on Viability of Eimeria bovis Sporozoites and LDH Release from MDBK Cells in the Presence of Sporozoites

Katrina Klobucher

University of New Hampshire

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Effect of Sodium Butyrate on Viability of *Eimeria bovis* Sporozoites and LDH Release from MDBK Cells in the Presence of Sporozoites

By

KATRINA NONNA KLOBUCHER

B.S. Animal Science, University of Massachusetts, Amherst, 2019

THESIS

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Master of Science

In

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This thesis/dissertation was examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Agriculture, Nutrition, and Food Systems: Agricultural Science by:

Thesis Director, Dr. Peter S. Erickson
Professor of Dairy Management and Extension Dairy Specialist
University of New Hampshire, Durham, NH

Dr. Thomas Foxall
Professor of Biological Science
University of New Hampshire, Durham, NH

Dr. Elizabeth Brock
Clinical Assistant Professor of Agriculture, Nutrition, and Food Systems
University of New Hampshire, Durham, NH

Dr. Andre F. Brito
Associate Professor of Dairy Cattle Nutrition and Management
University of New Hampshire, Durham, NH

On May 23rd, 2022

Approval signatures are on file with the University of New Hampshire Graduate School.
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Abstract

Supplementing Sodium Butyrate to Limit Fed Heifers: Effects on Growth, Health, Nutrient Digestibility and in vitro Coccidia Viability and Activity

By

Katrina Nonna Klobucher

University of New Hampshire, 2022

Two experiments were conducted to investigate the effects of sodium butyrate (SB) supplementation in heifer diets. The objectives of experiment 1 was to evaluate the growth, apparent total tract digestibility, and coccidia levels of post-weaned limit-fed heifers supplemented with SB. A 12 week randomized complete block experiment was conducted using 24 Holstein heifers. Treatments were 100g soybean meal (control; CON) and 0.75 g of SB/kg of body weight + 100g soybean meal (SB). Diets were formulated to contain 16.4 % crude protein (CP), 2.27 Mcal/kg metabolizable energy (ME), and fed at a rate of 2.15% of body weight on a dry matter basis. Supplementing SB resulted in animals that tended to be shorter in the withers and grow less per day in length. Average daily gain was greater by week for the SB treatment. Control heifers tended to have fewer coccidia oocytes per kg of feces by week. Plasma glucose concentrations were lower and blood ketone concentrations were greater for the SB treatment. Final urine volume was greater for SB heifers. No difference was seen on purine derivatives. The goal of Experiment 2 was to determine the viability of sporozoites from *Eimeria bovis in vitro* exposed to SB, monensin (MON), or butyric acid (BA) and to determine the effects of SB on sporozoite damage to bovine epithelial cells in comparison to MON. Results showed that BA and
MON decreased sporozoite percent viability. Sodium butyrate and MON decreased cell membrane damage in MDBK cells from sporozoites based on lactate dehydrogenase release.
Chapter I

Review of Literature

Introduction

By the year 2050 the world’s human population is expected to reach 9.9 billion people, an increase of about 25% over thirty years. Agriculture will be faced with an increasing demand to supply food to the worldwide population, increasing the need for efficiency of production. Agriculture contributes 5.2% to the total gross domestic product of the United States (USDA, 2019). In 2020, cash receipts for animals and animal products totaled $165 billion, with dairy receipts accounting for 24.6% or $40.5 billion (USDA, 2021). With the large percentage animal agriculture contributes, producers should continue to find ways to make their herds more efficient.

A healthy herd of cattle is the key to success and starts with knowledgeable and skillful management practices that contribute to better animal welfare and health. Proper management and nutrition of replacement heifers is shown to increase future profitability of the herd. It is estimated that it costs $2,241 to raise a heifer to 24 months of age, with feed costs accounting for about 50% of the total cost (Iowa State, 2019). While in the Northeast it is projected to be even higher to raise heifers. With costs so high, animals need to be raised in a way that reduces feed and labor costs, therefore increasing the efficiency of raising heifers. The way heifers are raised
is critical in today’s world for both economic reasons and the efficiency of resource usage. Weaning dry matter intake, health of the animal, and body weight at first calving have all been shown to affect first lactation milk production, while grain intake at a young age has an effect on lifetime milk production (Heinrichs and Heinrichs, 2011). Efficiently raised heifers calve when they are less than 24 months of age and produce 88% of a mature cow’s milk production. In contrast, inefficiently raised heifers, cost the producer $305 more per animal that calve 1.6 months later and produce 82% of what a mature cow on the farm is producing (Heinrichs et al., 2013). Heifers that are raised to gain over the targeted amount of 800 g/d - 900 g/d during the prepubertal time are shown to have a decreased first lactation milk yield (Zanton and Heinrichs, 2005). Consequently, to maximize profitability and production, research is being conducted to raise quality replacement heifers.

A fully developed rumen is needed to ensure that a heifer can grow to the best of her ability. The fermentation of solid feed and roughages begin this process to becoming a fully functioning ruminant. Microbes within the rumen begin to breakdown fiber and carbohydrates into volatile fatty acids (VFA). The primary VFA produced in the rumen include acetate, propionate, and butyrate. The type of feed a calf consumes establishes the predominant microbes that will grow within the rumen. By giving calves starter grain, starch fermentation can occur, dropping the ruminal pH due to the byproducts of fermentation. This slight drop in the pH allows bacteria to thrive and multiply. Most importantly, the consumption of starter grain leads to the formation of butyrate. Butyrate stimulates the growth of ruminal papillae, increasing the surface area for nutrient absorption, leading to a functional rumen and helping to develop ruminal musculature (Heinrichs and Jones, 2018).
The breakdown of carbohydrates and fiber leads to the formation of VFA. When feeding VFA salts including sodium acetate, sodium propionate, and sodium butyrate (SB) to two week old calves, a significant increase in development of the rumen mucosa was seen (Sander et al., 1959). Animals can digest feed and utilize nutrients more efficiently by providing sources for the rumen to increase its absorptive capacity. This in turn will increase feed efficiency (FE).

Antibiotic ionophores, such as monensin and lasalocid, have also been shown to increase FE (Heinrichs, 1993). Calves fed lasalocid in milk replacer (MR) had greater body weight gain and shed fewer coccidia oocysts than calves that were not fed an ionophore (Quigley et al., 1997).

The European Union banned the use of antibiotics as growth promoters in animal feeds in 2006 (European Commission, 2005). Since then, there has been pressure to find alternative feed additives that can provide similar results to ionophores. Research has been performed looking at the effects of SB on dairy cattle including calves, post-weaned heifers, and lactating cows. Sodium butyrate has been shown to stimulate growth and concentration of ruminal papillae in cattle and other beneficial results in different livestock species (Mentschel et al., 2001; Górka et al., 2009; Guilloteau et al., 2009; Górka et al., 2011; Kowalski et al., 2015; Górka et al., 2018). In broiler chickens, it has been shown to increase intestinal epithelial growth. Chickens that were heat stressed and fed butyric acid were found to have improved intestinal health and accelerated epithelial recovery (Abdelqader and Al-Fataftah, 2015). When the recovery of damaged tissue is improved, nutrient digestion and absorption that takes place in the small intestine can be preserved. With these results, the supplementation of SB into post weaned heifer diets is being investigated. These studies presented here focus on the supplementation of SB as a growth enhancer and anti-coccidial in vivo and the destruction of *Eimeria bovis* in vitro.
The Preweaned Ruminant

A very critical time in the life cycle of a dairy calf occurs when transitioning from abomasal digestion to ruminal fermentation. This shift begins with proper management and nutrition. It is essential to understand the nutrient requirements and digestive process of calves to ensure a successful transition to a true ruminant. If dietary requirements are not met early in the calf’s life it will hinder its future ruminal development and growth, therefore, reducing production potential.

Calves are born with a nonfunctional rumen thus relying on abomasal digestion. The first nutrients a calf receives consist of liquid feeds, starting with colostrum and switching to milk or MR. When gastric digestion is relied upon during the first few weeks of life, the calf utilizes the esophageal groove, a muscular fold of tissue, to shunt liquid feed past the reticulorumen and into the abomasum (Hegland et al., 1957). The abomasum breaks down the liquid feed to supply the calf nutrients. After day one of life, starter grain is provided allowing solid feed intake. Solid feed is fermented in the rumen and the end products, including VFA, are utilized for the development of the ruminal epithelium. By providing the correct nutrition, the development to a fully functional rumen occurs.

Choosing a MR program and how it is fed can improve calf performance. Chapman et al. (2016) performed a study using Holstein bull calves looking at MR programs and the effects on calf performance and digestion of nutrients with age. Three different diets were fed: conventional, moderate, and accelerated. The conventional MR program was 0.44 kg of dry matter of a 21% CP, 21% fat MR, fed for 42 d. The moderate diet was 0.66 kg of dry matter of a 27% CP, 17% fat MR, fed for 42 d. The aggressive treatment was 0.87 kg of dry matter of a 27% CP, 17% fat MR, fed for 49 d (Chapman et al., 2016). Conventionally fed calves had the least
average daily gain when compared to the other treatments, gaining 0.35 kg/d while, the moderate treatment gained 0.51 kg/d and accelerated 0.55 kg/d (Chapman et al., 2016). Accelerated fed calves grew well pre-weaning but post-weaning these results changed. Average daily gains dropped for the accelerated treatment calves from wk 5 to 7. During this period, average daily gain continued to increase for the conventional treatment as well as the moderate treatment. From wk 7 to 8 average daily gains of the accelerated fed calves began to increase. Calves of the accelerated treatment had decreased preweaning starter intake. Other studies have shown similar results (Hengst et al., 2012; Chapman et al., 2017). The calories needed for the calf were provided from MR, therefore the need for energy did not drive starter intake. This can lead to a reduction in ruminal development causing a reduction in ruminal fermentation. Calves on the accelerated treatment had lower digestibility of neutral detergent fiber (NDF) and acid detergent fiber (ADF), both of which are digested within the rumen of calves, showing how digestibility can change with diet. Based on this study it was found that feeding a high amount of MR can lead to lower digestibility of nutrients post-weaning. During the preweaning phase, to stimulate rumen development, starter grain must be consumed. Therefore, feeding high levels of milk or MR can lead to lower starter intake delaying ruminal development.

The supplementation of starter grain ensures the beginning development of the reticulorumen. At two weeks of age, calves will begin to consume large amounts of solid feed (Williams and Frost, 1992; Khan et al., 2008). Starter grain comes in many different physical forms which can affect intake and ruminal development (Lesmeister and Heinrichs, 2004; Bach et al., 2007; Suarez-Mena et al., 2015; Terré et al., 2015, 2016). Liquid feed intake can also effect starter intake (Khan et al., 2011).
Particle size of the calf’s diet can also impact ruminal function and digestive capacity. Beharka et al. (1998) tested the effect of particle size of a calf’s diet to see the effects on gastric development. Eighty bull calves were fed ground (1 mm particle size) or unground diets equal in composition (25% alfalfa hay and 75% grain mix). Calves were fed milk at a rate of 8% of body weight (BW) and starter adjusted based on consumption. At 10 weeks of age, calves were slaughtered, and all digestive organs were removed and weighed. Diet form had no effect on the weights of the reticulorumen or abomasum. However, when ruminal tissues were examined, they saw differences in shape, volume, and length of ruminal papillae among calves fed different diets. The animals on the ground diet had shorter, thicker, and more branched papillae, compared to calves on the unground diet, which had uniform, flattened, and tongue shaped papillae. Calves on the ground diet had less surface area even though the papillae showed signs of branching (Beharka et al., 1998). Branching may have occurred as a compensation for the loss of surface area and absorptive ability due to the incomplete maturation of the epithelium which also occurs in diets containing high levels of concentrates or easily fermentable components (Bull et al., 1965).

Based on the information presented, the following can be concluded about factors known to impact the development of the preweaned ruminant. With the reliance of liquid feed for the first few days of life, it is important to introduce solid feed early in a calf’s life. The solid feed will help to establish a functional ruminal environment. Feeding starter provides additional sources of energy and protein. The particle size of starter grain can impact the morphology of the ruminal papillae. Finely ground starters will negatively impact ruminal development producing shorter and branched papillae while an unground starter will produce papillae that are longer and more evenly distributed. Starter is fermented in the rumen producing VFA that are utilized by the
ruminal epithelium. Therefore, it is important to provide calves with the correct nutrition during the preweaning period to establish a well-developed and functional rumen.

**Weaning**

The time in a calf’s life when they transition from abomasal digestion of liquid feeds to fermentation of solid feeds is known as weaning. Producers will decide to wean their animals based on BW, age, solid feed intake, or a combination of factors. After weaning, animals will rely on solid feeds, except for water, as a source for all nutrients. It is estimated that 70% of calves are weaned at 7 weeks of age or later (Jones and Heinrichs, 2016). Producers that choose to wean earlier in life will do so to reduce feed and labor costs.

**Volatile Fatty Acid Production**

When carbohydrates are fed to cattle, anaerobic fermentation occurs in the rumen producing VFA. Depending on diet and time after feeding, concentrations within the rumen differ (Bergman, 1990). Microbial action in the rumen is crucial for VFA production. Microbes within the rumen digest structural carbohydrates which is performed by cellulolytic and hemicellulolytic bacteria and bacteria that will digest both cellulose and hemicellulose. Enzymes such as cellulases and hemicellulases are released in the rumen to digest structural carbohydrates. Oligosaccharides are created and can further be broken down into hexoses and pentoses, different units of carbohydrates. Bacteria can use these to create ATP leading to VFA production.

Pyruvate production in the rumen is essential for VFA production. Glucose will be converted to pyruvate though glycolysis. After glycolysis, depending on the ruminal
environment, the use of pyruvate can differ. The most common pathway for the creation of pyruvate is the Embden-Meyerhof-Parnas (EMP) pathway due to the high yield of ATP. Glucose can also be broken down into pyruvate through the pyruvate-ferredoxin oxidoreductase pathway. In this pathway glucose is converted to pyruvate, which is then broken down into acetyl coenzyme A (acetyl-CoA), CO₂, and reduced ferredoxin. The result of this pathway is acetate. Acetate can also be produced through the pyruvate-formate lyase system also creating one ATP molecule.

For butyrate to be formed in the rumen, two pyruvate molecules and three key enzymes are needed. The enzymes are phosphotransbutyrylase, butyrate kinase, and butyryl CoA:Acetyl CoA transferase. The driving factor for the conversion of glucose to butyrate is the recycling of the coenzyme A. During absorption through the ruminal epithelium, butyrate is converted into ketone bodies, causing low butyrate levels in the blood. Of the ketone bodies formed about 80% result in β-hydroxybutyric acid, with the other 20% being acetoacetate and acetone. Butyrate reaching the liver is rapidly metabolized.

There are two pathways for the formation of propionate: the randomizing pathway and the acrylate pathway. The amount of propionate produced depends on the ruminal environment and bacterial species present. During absorption, most propionate enters the portal blood with 2-5% being converted to lactic acid before absorption. Before reaching the liver, it is converted to glucose where it can act as a glucogenic precursor.

**Butyrate**

Ruminal development heavily relies on butyrate when compared to the other major VFA (propionate and acetate). Butyrate is formed when dietary carbohydrates, including cellulose,
hemicellulose, starch, and soluble sugars, undergo fermentation in the rumen. Butyrate that is formed is metabolized by the ruminal epithelium (Ash and Baird, 1973). Papillae growth in the rumen is stimulated by butyrate, causing an increase in surface area for absorption of nutrients (Sander et al., 1959). Sodium butyrate, a sodium salt, that can be fed in the diet can help to increase levels of ruminal butyrate, leading to more developed papillae in young animals (Sander et al., 1959). Promising effects have also been seen in pre-partum cows, SB increased papillae development allowing for better diet adaptation and nutrient absorption (Kowalski et al., 2015).

A well-developed rumen is very important in a young heifer’s life. Calves that are supplemented with SB show an increase in growth and gastrointestinal tract (GIT) development (Górka et al., 2018). With an increase in growth rates, heifers can reach breeding age earlier allowing producers to breed heifers sooner. When this occurs, heifers can enter the milking herd at an earlier age, allowing for a return on investment sooner. Efficiency of the farm would be increased through a reduction of feed costs and earlier enrollment in the milking herd.

**Sodium Butyrate**

Supplementation of SB has been seen to be beneficial at different life stages of cattle from birth to the dry period. Most research regarding SB has been focused on the supplementation to neonatal ruminants. Liu et al. (2019) looked at the infusion of SB in neonatal twin lambs. Lambs were assigned to receive an oral infusion of SB (0.36 g/kg of BW) or the same volume of saline starting at d 10 of age through d 49. At the end of the treatment period, lambs were slaughtered, and ruminal papillae thickness and morphology were measured. Lambs given infusions of SB had longer ($P = 0.02$) and wider ($P < 0.01$) papillae, leading to a greater surface area ($P < 0.01$) than the control group. Sodium butyrate infused animals had greater total
VFA concentration \((P = 0.02)\) than saline treated animals. Volatile fatty acids are the primary stimulator of ruminal development and energy source, therefore SB treated animals had a more developed rumen which is confirmed by the rumen morphology measurements.

Supplementation of butyrate in the liquid feed of calves lead to increased development of the GIT due to indirect and direct effects (Górka et al. 2018). Butyrate serves as an energy source for the GIT, acting as a direct effect. Indirectly, butyrate can affect the secretion of GIT peptides and hormones and stimulation of the vagal nerve (Górka et al. 2018). The low concentrations fed in the study by Guilloteau et al. (2009);(3 g/kg of DM) showed that the indirect effects are more important on development because low levels of supplementation would not provide an adequate source of energy for the digestive tract tissues. In this study, Guilloteau et al. (2009), compared the supplementation of SB and flavomycin (an antibiotic) in MR. Calves fed the SB treatment had greater BW gain \((P = 0.02)\) and feed conversion ratio \((P = 0.02)\) compared to the flavomycin group, which is thought to be due to an increase in the efficacy of digestive enzymes making digestion more efficient (Guilloteau et al., 2009). Sodium butyrate was not found in the blood of calves on this study therefore it must be acting at the GIT level rather than after absorption to help increase the efficiency of digestion (Guilloteau et al., 2009). Sodium butyrate influences gut regulatory peptides secretion by the stomach and intestine, but studies have failed to determine the exact indirect mechanisms (Guilloteau et al., 2009, Górka et al., 2018).

Górka et al. (2009) performed a study looking at the effects of SB when added to MR and starter compared to no supplementation in MR and starter. Papillae length and width were measured to observe ruminal development. The SB treatment had longer \((P < 0.01)\) and wider \((P < 0.01)\) papillae in the rumen when compared to the control group. Reticulorumen weight tended to be greater \((P = 0.13)\) in treatment calves compared to control calves. This could be due to SB
treated calves having a tendency for increased starter intake ($P < 0.10$) in the final week of the study. This increase in starter intake could have led to ruminal papillae development allowing for a more developed rumen and greater reticulorumen weight. It is possible that other VFA produced in the rumen helped to increase ruminal development as well (Górka et al., 2009). From the results of this study, it is unclear if the SB in the MR or starter resulted in these benefits.

More recent studies with SB look further into the effects on mode of delivery of SB to calves (Górka et al., 2011; Górka et al., 2014). In calves, milk is shunted past the rumen through the esophageal groove to the abomasum where it then travels down the rest of the GIT. Starter grain, however, will be brought into the rumen when a calf begins to consume solid feeds.

Górka et al. (2011) explored the method of delivery of SB on ruminal development. Animals were supplemented SB in starter grain and MR or just one of these two feed sources. The control treatment had no supplementation of SB in any feed source. Average daily gain ($P = 0.04$) and BW gain ($P = 0.09$) tended to be greater when MR was supplemented compared to non-supplemented MR. The supplementation in starter grain also resulted in positive effects including an increase in starter intake ($P = 0.05$) from d 15-21 compared to non-supplemented starter grain treatment animals. When animals were supplemented with SB in either MR or starter, they tended to be healthier. Animals whose starter was supplemented had a decreased number of days with electrolyte therapies over the entire study and a decrease in the number of days with scours compared to animals fed starter grain with no supplementation. Animals with supplemented milk replacer also tended to be healthier with a trend of fewer days with electrolyte therapy from d 0-7 and the tendency of positive effects to fecal consistency from day 8-14. When SB was supplemented to both the MR and starter grain improvements in ruminal
development were seen. Calves with supplementation in both solid and liquid feed had a greater reticulorumen weight as a percent of total BW \((P = 0.05)\) compared to other calves on the study. Papillae length \((P = 0.04)\) was greater and papillae width \((P = 0.09)\) tended to be greater when SB was supplemented to all feed consumed compared to other treatment groups. Based on these effects it was concluded that supplementation of SB to MR stimulates ruminal development indirectly while the supplementation to starter grain affects ruminal development directly (Górka et al., 2011).

Górka et al. (2014) used the same treatments as Górka et al. (2011) and researched small intestine development. The greatest effects on small intestine development were seen when MR was supplemented compared to starter grain. Supplemented MR increased cell proliferation and decreased cell apoptosis when compared to animals receiving non-supplemented MR. The epithelial structure was affected when milk replacer was supplemented, calves had shorter villus height in the proximal \((P = 0.02)\) and middle jejunum \((P = 0.04)\) when compared to animals fed non supplemented MR. Milk replacer can bypass the rumen leading to the small intestine, while starter grain cannot. Therefore, milk replacer can act on lower GIT development. It is also important to note that since newborn calves were used in this study, starter grain intake is very low through the first few weeks of life, unlike milk or MR. The results from this study suggested that the supplementation of SB to MR led to an enhanced maturation of the mucosal epithelium of the small intestine. Sodium butyrate has also been demonstrated to have antibacterial and anti-inflammatory effects (Guilloteau et al., 2009). Therefore, the results seen in this study can also be explained by this property of SB, enhancing the maturation of the small intestine.

More recently studies have been conducted looking into the effects of SB on post weaned heifers, twelve weeks and older (Rice et al., 2019; Stahl et al., 2020). Rice et al. (2019) looked at
various levels of SB supplementation in post weaned heifers. Treatments were: 100g of soybean meal carrier, 0.25g, 0.50, and 0.75 g of SB/kg of BW plus carrier. Average BW increased linearly as well as final BW tended to increase as SB concentration increased. It was also observed that FE tended to increase ($P = 0.08$) as SB increased. This study also found SB has the potential to decrease coccidia. A quadratic effect ($P = 0.03$) was seen on coccidia prevalence.

Heifers being fed 0.25 g of SB/kg of BW had the least amount of coccidia oocysts. Stahl et al. (2020), also looked at the supplementation of SB to post weaned heifer, twelve weeks and older. Monensin was used as a treatment to see its effects compared to SB. The four treatments were as follows: 100 g soybean meal carrier (control), 0.75 g SB/kg of BW + carrier, 1 mg MON/kg of BW + carrier, and 1 mg of MON/kg of BW + 0.75 g of SB/kg of BW + carrier. Similar results were seen compared to Rice et al. (2019). Heifers fed an additive tended to have a greater average BW compared to the control ($P = 0.10$). Dry matter intake was greater for heifers fed an additive compared to a control ($P = 0.03$). Additives also influenced coccidia prevalence. The number of coccidia oocysts was reduced for heifers fed an additive compared to the control ($P = 0.03$) and the incidence of coccidia was also reduced ($P < 0.01$) when heifers were supplemented with an additive compared to the control group. Additives increased growth and reduced fecal coccidia counts compared to the control. Sodium butyrate had similar effects to monensin.

In lactating dairy cows, butyrate is an important VFA associated with energy. Butyrate is absorbed through the rumen wall and transformed into a ketone body to be used as an energy source. Ketone bodies can be stored to use for milk fat synthesis. Izumi et al. (2019), looked at the effects of butyrate supplementation on DMI, milk production, and blood metabolites to lactating cows fed varying levels of starch. Animals supplemented with butyrate received a
mixture of 70% SB and 30% fatty acid mixture at 2% of dietary dry matter. The control group received 70% wheat bran and 30% fatty acid mixture. The inclusion of butyrate did not affect DMI. but did affect milk fat content. Cows receiving SB had an increased milk fat content ($P = 0.04$) and increased milk fat yield ($P = 0.02$) compared to animals not supplemented with SB. Sodium butyrate also tended to increase 4% fat-corrected milk yield ($P = 0.08$) and 4% fat-corrected milk yield/DMI ($P = 0.08$) compared to the control group. Sodium butyrate supplemented animals had decreased milk urea nitrogen levels ($P = 0.02$) compared to the control. Lastly, serum $\beta$-hydroxybutyrate concentration was increased ($P < 0.001$) when supplemented with SB compared to the control. These levels did not exceed 1.2 mM which would have resulted in subclinical ketosis. Overall, the inclusion of SB in the diet of lactating cows increased milk fat components regardless of starch level in the diet (Izumi et al., 2019).

Although further research should be conducted on this topic, this study provides strong evidence that supplementing butyrate such as SB could be beneficial for lactating ruminants.

Nutrition during the dry period is critical to reducing the incidence of many different metabolic issues. Research has been done to investigate the effect of feeding SB to dry cows. Kowalski et al. (2015), used two treatments, without SB or with 300g SB/day, to see the effect of SB during the 30 days prior to the expected calving date. After calving, cows were monitored to 60 d in milk. In the 5 d before parturition, cows being fed SB consumed 1.7 kg of DM/d more than the control diet (Kowalski et al., 2015). It has been seen in previous studies that the supplementation of SB to newborn calves can increase ruminal papillae length and development (Guilloteau et al., 2009; Górka et al., 2009; 2011; 2018). Dairy cows during the pre-fresh period are undergoing a change in ruminal papillae due to dietary changes. Moderate to low starch diets are commonly fed during this time to reduce the risk of acidosis and other common metabolic issues.
issues. These diets do not allow for adequate ruminal papillae growth and development for when a cow freshens and begins to consume a lactating cow diet. Therefore, the inclusion of SB can help promote ruminal papillae development along with the increase in DMI compared to the control. An increase in DMI can help to reduce the prevalence of metabolic disorders during the fresh period. Overall, the supplementation of SB throughout the life of a cow can be very beneficial on growth promotion, DMI, ruminal development, and coccidia prevalence.

Limit Feeding

Precision feeding, also known as limit feeding, is a management technique used by some producers when feeding heifers. The provided feed intake of heifers is restricted to a percentage of BW and therefore nutrient requirements are more precisely met due to limited intake. Feed efficiency is a management concept used for lactating dairy cows (kg milk/kg feed). In heifers, FE is measured as kilograms of gain per kilograms of feed (Heinrichs and Zanton, 2016). Having efficient animals on the farm is key to be successful economically. Highly digestible feeds used allow for greater FE, reducing manure output and decreasing the overall cost of raising heifers.

Diet Formulation: Energy

Energy requirements of heifers change over time based on size, growth rate, and environment (Heinrichs and Zanton, 2016). Limit fed diets tend to have higher ME concentration, with the energy requirement being precisely met. This is opposed to feeding energy ad libitum and allowing the animals to select her own energy consumption. Overall, energy should be fed to allow for 0.8-0.9 kg of average daily gain.
**Diet Formulation: Protein**

A balanced diet is essential when limit feeding. A total mixed ration (TMR) is fed with no additional sources of free choice forage. When formulating diets, CP should be balanced for primarily before other nutrients (Heinrichs and Zanton, 2016). Pre-pubertal heifers should receive 14-15% CP based on 1.15% BW DMI/d. As heifers age to become post pubertal, they should receive 13-14% CP based on 1.65% BW DMI/d. Throughout the heifer’s life, soluble protein should be maintained at 30-35% of the diet. Total protein is just as important as protein fractions for heifers. Added rumen undegradable protein (RUP) is of little value to the heifer due to the inability to breakdown in the rumen. Soluble protein or rumen degradable protein (RDP) are efficiently used by heifers, allowing for sufficient rumen microbial protein production throughout the day, despite the limited intake (Heinrichs and Zanton, 2016).

**Management Aspects of Limit Feeding**

Limit feeding comes with different management techniques when compared to the conventionally fed (*ad libitum*) heifer. Because feed levels are based on a percentage of BW all heifers must be weighed once a month. Heifers should be weighed at the same time of day to account for gut fill. This allows average daily gain to be calculated. As a benchmark, heifers should gain 0.8-0.9 kg per day. Weighing animals will also determine groups of animals. In a limit feeding situation animals should be housed in groups of similar age and size, up to a 90 kg difference between animals within a group (Heinrichs and Zanton, 2016). The size of the group must also be considered when creating groups of animals. Since all feed is consumed shortly after feeding, each animal needs to access the feed bunk at once. Within the pen, different types of bedding need to be considered. In a precision fed diet, animals are achieving energy
requirements not by gut fill limiting DMI, therefore gut fill is not achieved. Animals will have the tendency to consume edible bedding such as straw and wood shavings. Alternatives such as mattresses or sand can be used. Animals can be limit fed until the prefresh period, as there are no adverse effects of limit feeding on calf birth weight, dystocia, metabolic problems, early lactation intakes, or milk production (Heinrichs and Zanton, 2016). During the prefresh period, the rumen and gut volume change rapidly. Overall monitoring of heifers is important when implementing limit feeding to ensure quality herd replacements.

**Ionophores**

Ionophores were introduced into the dairy cattle industry in the United States in the 1970’s. Ionophores are polyether antibiotics with no known ruminal antibiotic resistance (McGuffey, 2017). Ionophores affect Gram-positive bacteria in the rumen. The ion gradient in the cell membrane is disrupted, reducing the available K+ and ATP preventing cell growth (Russell and Strobel, 1989). Gram-negative bacteria are able to proliferate under the use of an ionophore due to thicker cell membranes causing more propionate to be made in the rumen (Callaway et al., 2003; Gupta, 2017). Ionophores were introduced to the broiler industry for the prevention and control of coccidiosis (McGuffey, 2017). Monensin (MON) and lasalocid are popular ionophore choices among dairy producers. Monensin works to limit coccidia growth by disrupting the movement of sodium and potassium ions. Potassium ions leave the cell while hydrogen ions enter, the cell then tries to maintain homeostasis but runs out of energy and ruptures (Gupta, 2017).

In a study done by Baile et al. (1982), MON was supplemented to heifer diets, at differing levels, 0, 200, or 600 mg per day. Sixty heifers were supplemented starting when they
weighed 196 kg until 3 d prior to calving. Animals supplemented with MON gained an additional 0.09 kg/d when compared to the control heifers. Monensin also lowered DMI, leading to greater FE. Ionophores help to increase FE, weight gain, and health, reducing morbidity and mortality (McGuffey, 2017).

Coccidiosis

Coccidiosis is a common disease found on dairy farms that can cause significant economic losses for producers. An economic loss can be seen due to the treatment cost as well as animals being setback from their herd mates due to intestinal damage. Mortality can also occur when severe cases are seen. Coccidiosis is caused by the protozoan species *Eimeria*. There are many species of *Eimeria*, twelve of which have been identified in the feces of cattle. Of the twelve, *E. zuernii*, *E. bovis*, and *E. auburnensis* are most commonly associated with the clinical disease (Constable, 2015).

Animals one month in age to a year are most susceptible to infection. Coccidiosis does not occur in the first three weeks of life due to its life cycle; therefore, it is not associated with the neonatal diarrhea complex (Constable, 2015). The onset of diarrhea occurs 16-23 d after infection. The disease is sporadic throughout the year and seasons with wet weather and severe temperature fluctuations causing a higher presence in the environment. Other causes of stress including overcrowding and pen changes can also cause an increase in disease presence (Matjila and Penzhorn, 2002; Jolley and Bardsley, 2006). Coccidiosis due to stress is mediated by increased corticosteroids circulating through the body (Eness, 1985). Animals may present with subclinical coccidiosis, meaning oocysts are present but animals appear healthy and undiagnosed. In cases like these, FE is reduced, costing the producer. Symptoms of coccidia
include fever, decreased appetite, weight loss, and dehydration. Symptoms of more chronic infections include bloody diarrhea continuing for greater than a week or thin feces with shreds of epithelium and mucus (Constable, 2015). Coccidiosis causes enteritis, inflammation of the large intestine, and damage to the mucosa of the lower small intestine (Constable, 2015). This intestinal damage hinders nutrient absorption leading to a decrease in DMI, a key component to growth of young animals.

The life cycle of *Eimeria* starts in the environment (Figure 1). An infected animal will shed non-sporulated oocysts. These oocysts become sporulated in the environment with the presence of oxygen and then are infective (2). Animals ingest the sporulated oocysts from feces, in the environment, or other sources. Once in the gut, sporozoites are released from the oocyst and can reproduce through asexual reproduction (3). Sporozoites can invade and infect digestive cells becoming merozoites. First generation merozoites invade neighboring cells to multiply (4). Merozoites differentiate into gametes which then produce oocytes that are shed in the feces (5/6) (Pié Orpí, 2020).
Figure 1. Emieria Life Cylce (Pié Orpí, 2020).

In vitro methods can be used to test the efficacy of treatments and preventatives. This is performed by harvesting oocysts from feces of affected animals. Oocysts are harvested through flotation using a concentrated sugar solution. Debris is removed from the oocysts using different techniques. Sporulation can be mimicked using aeration in an aqueous solution. Once sporulated and sterilized, sporozoites can be released through enzymatic processes (Kurth and Entzeroth 2008; López-Osorio et al. 2020). The resulting sporozoites can be used to infect cells in vitro.

Conclusion

With the goal to raise quality replacements for the milking herd, producers also look for ways to do so more efficiently. This could be through increased FE, BW, or overall health of the animal. Supplements to a heifer’s diet can help to achieve the farms goals. Commonly antibiotics are fed, but with the current move to feed less of them, alternatives need to be researched. Studies conducted by Rice et al. (2019), and Stahl et al. (2020), investigated the supplementation of SB to post weaned heifers on growth and health performance. The current studies continue the research on SB being included in the diets of post weaned heifers as well as E. bovis activity in vitro in the presence of SB.
Chapter II

Supplementing Sodium Butyrate to Limit-Fed Heifers: Effects on growth, health, and nutrient digestibility

Abstract

The objective of this study was to assess the growth, apparent total tract digestibility of nutrients, and prevalence of coccidia in post-weaned heifers when limit fed a diet supplemented with sodium butyrate (SB). A 12 wk randomized complete block experiment was conducted using 24 Holstein heifers [92.8 d ± 1.9 d of age and initial body weight (BW) of 106.18 kg ± 19.46 kg (mean ± SD)]. Treatments were 100g soybean meal (control; CON) and 0.75 g of SB/kg of BW + 100g soybean meal (SB). Diets were formulated to contain 16.4 % crude protein (CP), 2.27 Mcal/kg metabolizable energy (ME), and fed at a feed out rate of 2.15% of BW on a dry matter basis. Intakes were recorded daily while growth measurements and BW were recorded weekly. Urine and fecal samples were taken biweekly. On d 42 through d 49 an apparent total tract digestibility phase took place using acid detergent insoluble ash as a marker. Growth measurements were similar among treatments except CON heifers grew longer and had a trend for them to be taller in the withers. A trend was observed for CON animals to have lower levels of coccidial oocytes by week. Heifers fed SB had lower blood glucose levels and higher levels of BHBA in the blood. Final urinary volume was higher for SB heifers and a trend was observed throughout the 12 wk study. These results suggested no growth benefit of supplementing SB to limit fed heifers.


Introduction

One of the largest expenses on a dairy farm is raising replacement heifers. Cutting costs associated with heifers is challenging without compromising the quality of animals raised. Feed, reproduction, and health related costs including treating for disease are all costly expenses. A balance must be found to reduce costs and still ensure animals will reach maturity at a younger age.

One management technique that can reduce costs is precision or limit-feeding. This feeding style can decrease feed costs due to a lower quantity of feed being offered. In a limit-fed diet, the ration is more nutrient dense, therefore feed is offered at a percentage of BW, and nutrient requirements are met more precisely. Animals can utilize more of the nutrients included in the diet, increasing feed efficiency (FE) which in turn reduces feed costs.

Feed additives, such as ionophores, can have many benefits when included in the ration. These benefits include increased FE, increased growth, and better overall health (Baile et al., 1982; Quigley et al., 1997). Feeding ionophores has been shown to improve overall health through reduced coccidian oocyst shedding (Quigley et al., 1997). However, in the European Union, there is a ban on the use of ionophores in feed (European Commission, 2005). In other parts of the world there continues to be a movement towards using fewer antibiotics in feed.

Sodium butyrate (SB) has been shown to have similar benefits to ionophores. These benefits have been seen through a more developed rumen, leading to an increase in papillae length and volume when calves are supplemented with SB (Górka et al., 2011a,b). This allows for the absorptive capacity to be increased within the rumen, utilizing more nutrients in feed (Górka et al., 2011a,b). Within the gastrointestinal tract (GIT), there has been shown to be an enhancement in the maturation of the small intestine epithelium when SB is included in milk.
replacer (Guilloteau et al., 2009; Górka et al., 2014). Sodium butyrate can also decrease cell apoptosis and increase cell proliferation in the small intestine (Górka et al., 2014). Pancreatic secretion is also shown to be increased when SB is fed, which aids in digestion (Guilloteau et al., 2010).

When supplemented with SB, post weaned heifers have also been shown to have positive effects such as an increase in BW, a tendency for greater FE and final BW, and reduction of coccidia oocysts when SB was included in heifer diets in different concentrations (Rice et al., 2019). Stahl et al. (2020), showed that SB was similar to monensin. Heifers were supplemented with SB, monensin, or a combination of both. When fed an additive animals tended to have greater average body weight, increased DMI, and a reduction in the prevalence of coccidia oocysts compared to the control animals (Stahl et al., 2020).

The objective of this study was to evaluate SB supplementation to limit fed post-weaned heifers on growth performance and health. The hypothesis of this experiment was that SB in a limit-fed heifer diet will increase growth, apparent total tract digestibility, and decrease coccidia.

Materials and Methods

Experimental Design and Treatments

This experiment was reviewed and approved by the University of New Hampshire Animal Care and Use Committee (Protocol No. 210201).

Twenty-four Holstein heifers with a mean age of 92.8 d ± 1.9 d (mean ± SD) and average initial BW of 106.18 kg ± 19.46 kg (mean ± SD) were blocked by date of birth and
randomly assigned to 1 of 2 treatments in a randomized complete block design. A power test was performed to determine correct sample size using results from ketone data from Rice et al. 2019. A sample size of n=10 was determined to detect significance. Treatments were: (1) carrier, 100g soybean meal (control; CON); (2) 0.75 g of SB/kg of BW + 100g soybean meal (SB). The average initial BW of treatment groups were: 106.1 ± 3.1 kg (CON) and 106.3 ± 3.1 kg (SB); (mean ± SE). Two heifers were treated for coccidiosis according to farm protocols with Amprolium (Corid, Huvepharma, Peachtree City GA) as a drench. Both animals were on the SB treatment. One heifer from the SB treatment was removed from the study due to severe coccidiosis and salmonella infection. One heifer was added to account for the animal removed from the study. This caused two incomplete blocks in the study. Each treatment had twelve heifers complete the study. All treatments were adjusted weekly according to individual BW, excluding control animals who received 100g of soybean meal daily throughout the study. The SB provided was unprotected and was a 90% SB product with 68-69% butyric acid and approximately 21-22% Na+ and 10% maltodextrin (Ultramix GF, Adisseo Inc. USA, Alpharetta, GA). Heifers entered the pen to train to use Calan doors (American Calan Inc.) at 12 wk of life. The study began on the first Tuesday of the 13 wk of age. Heifers stayed on the experiment for 12 wk.

Management and Feeding

Heifers were group-housed in a naturally ventilated freestall barn with mattresses with no bedding provided. Three adjacent pens (pen 1: 5.46 x 4.75 m; pen 2: 5.54 x 4.88 m; pen 3: 6.32 x 4.8 m) were utilized. Pen 1 having the capacity to hold 6 heifers, pen 2 having the capacity to hold 8 heifers, and pen 3 having the capacity to hold 8 heifers. Automatically refilling water
troughs allowed for free access to water throughout the study. There was no competition for stall space. Each heifer was given a training period that lasted an average of 9 d to train to the Calan doors (American Calan Inc., Northwood, NH).

Heifers were limit fed TMR (Table 1) at approximately 0700 h daily in individual feed tubs to allow for daily feed intake measurements. Feed was mixed and distributed using a motorized feeding vehicle (Super Data Ranger; American Calan Inc.). The ration was fed to allow for consumption of 2.15% of BW on a dry matter basis. The diet was formulated to have ME = 2.27 Mcal/kg and CP = 16.4%. The amount fed was adjusted weekly according to individual BW. Body weight measurements were taken Monday afternoons and amounts fed were adjusted for the following morning feeding. Treatments were top dressed and hand-mixed into each individual feed tub. If refusals were present, it was recorded, and a sample was taken. Orts were only seen in two heifers throughout the study when they were being treated for coccidia.

Feed Analysis

Feed offered to each heifer was measured daily at the time of feeding to determine dry matter intake (DMI). Samples of TMR were collected daily and composited by week. When orts were present, a sample was collected and measured to determine DMI. Both TMR and refusal samples were frozen at -20°C for future analysis. Samples were thawed overnight and placed in a forced hot air convection oven (Binder, Bohemia, NY) to dry at 55°C for 48 h for determination of DM.
Dried samples were ground through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) and sent to a laboratory for nutrient analysis (Dairy One Forage Laboratory, Ithaca, NY). Feed samples were analyzed for the following: Acid detergent fiber (method 5 in an Ankom Fiber Analyzer A2000; Ankom Technology; method 973.18, AOAC International, 1998), neutral detergent fiber (method 6 in an Ankom Fiber Analyzer A2000 with αamylase and sodium sulfite; Ankom Technology, Fairpoint, NY; solutions as in Van Soest et al., 1991), starch (YSI 2700 SELECT Biochemistry Analyzer; YSI Incorporated Life Sciences, Yellow Springs, OH), crude fat (ether extraction; AOAC 2003.05; AOAC International, 2006), ash (AOAC Method 942.05; AOAC International, 2006), and CP (AOAC method 990.03; AOAC International, 2006).

**Measurements and Blood Sampling Analysis**

Body weight and skeletal measurements were taken weekly at 1330 h every Monday throughout the study. Heifers were measured for body length, heart girth, and paunch girth using a weigh tape. Animals were also measured for withers height and hip height using a sliding scale height stick with bubble level. Heifers were weighed on a portable scale (Tru-Test™ EziWeigh5i, Uniontown, PA).

Blood samples were obtained from the jugular vein using a 20-gauge needle before the time of measurements for the duration of the study. Samples were collected in 2, 10 mL vacutainer tubes, one containing anticoagulant EDTA and the other without an anticoagulant (Monoject, Covidien Ilc., Mansfield, MA). Blood ketone concentrations were obtained using a hand-held electronic blood glucose and ketone monitoring device (Nova Max Plus, Nova
Biomedical, Waltham, MA; Deelen et al., 2016). Ketone concentrations were determined by taking a whole blood sample, not containing EDTA, and transferring it to the sensor of a test strip using a disposable pipette. Blood ketone levels were run in duplicate. Samples with EDTA were placed on ice until they were centrifuged at 1,278 × g at 4°C for 20 min (5430R, Eppendorf, Hamburg, Germany). Plasma was frozen at −20°C until further analysis. Plasma was thawed and plasma glucose concentrations were measured in duplicate via Wako Autokit for Glucose (Wako Diagnostics, Mountain View, CA) and read on a UV-visible spectrophotometer at a wavelength of 505 nm.

**Digestibility Measurements**

Each of the 24 heifers underwent apparent total-tract nutrient digestibility phase on d 42 on the study until d 49. Total mixed ration samples were taken Tuesday through Saturday and composited over 5 d. Total mixed ration samples were then frozen at -20°C for future analysis. Samples were thawed and placed in a forced hot air convection oven to dry at 55°C for 48 h to determine DM. Fecal grab samples were collected on Friday, Saturday, Sunday, and Monday every 12 h to represent a 24-h period (d 4: 0200 and 1400 h; d 5: 0500 and 1700h; d 6: 0800 and 2000 h; d 7: 1100 and 2300) by stimulating defecation or collecting feces directly from the rectum. Fecal samples over the 4-d period were combined to obtain a single composite and frozen at −20°C. Fecal samples were thawed at room temperature and emptied into aluminum trays to be dried in a forced-air oven at 55°C for 72 h until dried. The dried TMR and fecal samples were ground through a 1-mm screen Wiley mill (Thomas Scientific, Swedesboro, NJ). Ground samples were sent to Dairy One Forage Laboratory (Ithaca, NY) for analysis. Feed and
fecal samples were analyzed for acid detergent insoluble ash (ADIA) according to Van Keulen and Young (1977), and CP, NDF, ADF, starch, ash, and fat as previously described.

The equation used to estimate digestibility was: 100 - [100 x (% ADIA in DM consumed / % ADIA in feces) x (% nutrient in feces / nutrient consumed DM)].

**Coccidia Enumeration**

Fecal samples were obtained from each heifer prior to the start of treatment and then biweekly for the entire study. Samples were taken by stimulation or directly from the rectum from each heifer on Monday at 1330 h and analyzed for coccidian oocysts following the modified Wisconsin sugar fecal worm egg flotation method (Bliss and Kvasnicka, 1997). Heifers were observed daily for scours.

**Urine Analysis**

Urine samples were collected from each heifer before the start of treatment and biweekly thereafter through direct stimulation of the pudendal nerve. Samples were immediately brought to the lab where 8.4 mL of the sample was deposited into a centrifuge tube containing 32 mL of 0.072 N H$_2$SO$_4$ and frozen for later analysis.

Samples from the covariate, wk 6, and wk 12 were thawed at room temperature before analysis for creatinine, allantoin, and uric acid. Samples were analyzed colorimetrically for creatinine (assay kit # 500701, Cayman Chemical C., Ann Arbor, MI) and uric acid (QuantiChrom Uric Acid Kit DIUA-250, Hayward, CA) using a microplate reader (Epoch Bio
Tek Instruments, Inc., Winooski, VT) set at a wavelength of 490 and 590 nm respectively.

Allantoin (Chen and Gomes, 1992) was also analyzed colorimetrically using a UV/visible spectrophotometer (Genesys 10S Vis, Thermo Scientific) set at a wavelength of 522nm. Urine volume was estimated from concentration and excretion of creatinine using a regression equation by Chizzotti et al. (2008), for growing heifers. The equation used was: Creatinine excretion /kg BW = 0.28 ± 0.01– 0.000097 ± 0.000015 × BW. Excretion of total purine derivatives (PD) was calculated by the addition of allantoin and uric acid excretion.

**Statistical Analysis**

Initial measurements of BW, skeletal measurements, glucose, ketones, PD, and coccidia counts were used as covariates for their respective variable. Weekly measurements of DMI, ADG, FE (ADG/DMI), BW, skeletal measurements, coccidia counts, glucose, ketones, and PD were analyzed as a randomized complete block design with repeated measures using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) according to the following model:

\[
Y_{ijkl} = \mu + B_i + Trt_j + W_k + \beta X_{ij} + TrtW_{jk} + E_{ijkl}
\]

where \(Y_{ijkl}\) = the dependent variable; \(\mu\) = the overall mean; \(B_i\) = the random effect of block \(i\) \((i = 1, \ldots, 13)\); \(Trt_j\) = the fixed effect of the \(j\)th treatment \((j = \text{control, SB})\); \(W_k\) = the fixed effect of the \(k\)th week on study \((k = 1 \ldots 12)\); \(\beta\) = the regression (covariate coefficient); \(X_{ij}\) = the covariate measurement; \(TrtW_{jk}\) = the fixed interaction between the \(j\)th treatment and the \(k\)th week; and \(E_{ijkl}\) = the residual error \(\sim N(0, \sigma^2_e)\). The random effect of heifer within block subclass was used as the error term for the effect of treatment. The error within heifer across time was the residual error. It represents errors for repeated measurements in the experimental units (heifer). For all variables five covariate structures were analyzed, compound symmetry, first order-
autoregressive, Toeplitz, unstructured, and variance components. The covariate structure that resulted in the smallest Bayesian information criteria was used. Most variables were modeled using variance components covariance special structure. Coccidia/kg of feces was modeled using unstructured covariance spatial structure. Urinary volume was modeled using the compound symmetry covariance spatial structure. Paunch grith was modeled using the first-order autoregressive covariance spatial structure. The Kenward-Roger approximation option of the MIXED procedure of SAS was used to calculate the degrees of freedom. When covariate \( P \)-values were greater than 0.25 they were removed from the model.

Final urinary creatine excretion/ d results were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc.) according to the following model:

\[
Y_{ijk} = \mu + B_i + Trt_j + \beta X_{ij} + E_{ijk}
\]

where \( Y_{ijk} \) = the dependent variable; \( \mu \) = the overall mean; \( B_i \) = the random effect of block \( i \) (\( i = 1, \ldots, 13 \)); \( Trt_j \) = the fixed effect of the \( j \)th treatment (\( j = \) control, SB); \( \beta \) = the regression (covariate coefficient); \( X_{ij} \) = the covariate measurement; and \( E_{ijk} \) = the residual error \( \sim N (0, \sigma^2 e) \).

Final and initial measurements, overall gains, and apparent tract-total digestibility were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc.) according to the following model:

\[
Y_{ijk} = \mu + B_i + Trt_j + E_{ijk}
\]

where \( Y_{ijk} \) = the dependent variable; \( \mu \) = the overall mean; \( B_i \) = the random effect of block \( i \) (\( i = 1, \ldots, 13 \)); \( Trt_j \) = the fixed effect of the \( j \)th treatment (\( j = \) control, SB); and \( E_{ijk} \) = the residual error \( \sim N (0, \sigma^2 e) \).
Significant treatment effects were determined significant when \( P \leq 0.05 \) and trends at \( 0.05 < P \leq 0.10 \). All data points greater or lesser than 2.5 SD away from the mean were considered outliers and removed. The Univariate procedure of SAS 9.4 (SAS Institute Inc.) was used to evaluate normal distribution of data.

**Results**

Nutrient analysis of the TMR throughout the 9 mos trial is shown in Table 2. Intake and performance as described by skeletal measurements, DMI, FE, ADG, and BW are presented in Table 3. Throughout the study two animals were treated for coccidiosis. Both animals were treated with an Amprolium (Corid, Huvepharma, Peachtree City, GA) drench and were on the SB treatment. Both were treated due to coccidiosis.

There were no differences in average BW, ADG, initial BW, and final BW across the two treatments. Average BW \( (P = 0.04) \) and ADG \( (P < 0.01) \) was significant by week. Withers height \( (P = 0.06) \) tended to be greater and body length gain \( (P = 0.03) \) were greater for CON treatment compared to SB treatment. No other skeletal measurement differences were seen. Overall gains are reported in Table 4 and no difference was observed between treatments.

Coccidia and blood parameters results are presented in Table 5. No difference was observed between treatments for initial coccidia counts/ kg of feces \( (P = 0.88) \), coccidia counts/ kg of feces \( (P = 0.61) \), and coccidia rate of incidence \( (P = 0.25) \). Rate of incidence was determined by if any oocysts were present in the feces. Coccidia counts/ kg of feces \( (P < 0.10) \) tended to be greater by week for the SB treatment. Blood glucose concentrations \( (P < 0.10) \) were greater for animals on the CON diet. Final glucose concentrations \( (P = 0.09) \) tended to be greater
for CON heifers. Average ketone concentrations ($P < 0.0001$) were greater in heifers on the SB treatment when compared to the CON. Final ketone concentrations ($P < 0.01$) were also greater for animals on the SB treatment compared to heifers on the CON treatment.

Purine derivative excretion and urine volumes are shown in Table 6. No differences were observed for purine derivatives. Heifers supplemented with SB had a greater urine volume per day ($P = 0.01$) compared to the control heifers. Final urine volume for SB heifers was greater ($P = 0.04$) than heifers receiving no supplement.

The results from the apparent total tract digestibility are presented in Table 7. Animals supplemented with SB had greater digestibility of ADF ($P = 0.04$) and OM ($P = 0.04$). There was a tendency for digestibility to increase when heifers were supplemented with SB for the following nutrients: DM ($P = 0.05$), CP ($P = 0.07$), NDF ($P = 0.06$), and ash ($P = 0.1$).

**Discussion**

Previous research in supplementing SB to heifer diets contradict the present study. Rice et al. (2019), observed an increase in BW, and a tendency for greater FE and final BW when heifers were supplemented SB in increasing concentrations. Stahl et al. (2020), also observed many benefits to supplementing additives, either supplementing SB, MON, or the combination to heifer diets. These researchers found that when heifers were fed an additive, they tended to have a greater average BW and increased DMI. Heifers on the current study were limit fed compared to the conventional way of feeding heifers targeting at 10% orts. The following equation was used to calculate the amount fed to each heifer weekly, amount fed = (BW x 2.15%) / % DM of feed. Pre-pubertal heifers are suggested to have 2.15% BW of DMI/d (Heinrichs and Zanton,
Orts were only seen in two heifers when they were being treated due to coccidiosis. Due to BW being included in the calculation for the amount fed to limit fed heifers, DMI could only increase if BW increased. No effect of DMI was seen in the present study. These data are similar to others based on limitations to intake on limit fed heifers (Hoffman et al., 2007). Hoffman et al. (2007), limited intake on heifers and saw a linear decrease in DMI due to the nature of the feeding approach used. Feed efficiency is expressed as ADG/DMI in the current study. With no difference seen in ADG or DMI, FE was the same across treatments. Feed efficiency in the current study for the SB treatment was 0.29, Rice et al. (2019), had a similar result, observing 0.28 for the 0.75 mg SB/kg BW treatment. However, Stahl et al. (2020), observed a FE result of 0.25 for the SB treatment. This is supported by Pino et al. (2018), observing ad libitum fed animals to have a lower feed efficiency response, therefore limit fed animals have improved feed efficiency. Average daily gain had a treatment by week interaction, due to the strong significance of week in the present study. This same result occurred for average BW. Final BW was the same across treatments. Rice et al. (2019), saw a linear trend for final BW to increase when supplementing SB, Stahl et al. (2020), also saw a trend for final BW to be greater when supplementing SB, MON, or the combination compared to no additive. Studies from Hoffman et al. (2007) and Manthey et al. (2016), used the limit feeding strategy and observed no difference in growth of heifers compared to other limit fed heifers, similar to the present study. Supplementing SB resulted in a trend for daily withers height gain, and length gain to be lower. Overall gains were the same among treatments.

Rate of passage of nutrients through the digestive tract slows for animals who are limit fed (Zanton and Heinrichs 2008; Pino et al., 2018). These researchers showed that retention time in the rumen increases with limit fed diet and decreases in ad libitum diets. Based on the higher
level of ketones in the blood observed in this study, it is assumed that SB is dissociating in the rumen. Sodium ions are ionically bound to butyrate, in the rumen this bond is broken, allowing for the epithelium to convert the butyrate molecule to ketone bodies (Holtenius and Holtenius, 1996; Müller et al., 2002; Herrick et al., 2017; Rice et al., 2019; Stahl et al., 2020). During the phase from pre-ruminant to ruminant there is a change from glucose absorption in the intestines to gluconeogenesis in the liver (Baldwin et al., 2004). Sodium butyrate increases hepatic enzymes resulting in less carbohydrate available for post ruminal digestion leading to a decrease in glucose absorption (Rice et al., 2019; Stahl et al., 2020). These researchers saw a reduction in plasma glucose which is consistent with the present study. It is also consistent with lactating cow studies when butyrate or SB was infused (Huhtanen et al., 1993; Herrick et al., 2017).

No effect of treatment was seen in the present study on coccidian oocysts. This contradicts other studies when SB was supplemented to heifers (Rice et al., 2019; Stahl et al., 2020). It is thought, due to the assumed decreased rate of passage in heifers of the current study, no element of the SB molecule was reaching the SI to reduce inflammation, heal intestinal cells, or cause merozoite destruction as seen in the other studies.

Throughout the study no differences in PD were observed. Since the covariate, wk 6 and wk 12 were the only samples analyzed, not enough data may be present to have results observed. Purine derivatives have a direct relationship to microbial protein synthesis and is a non-invasive way to estimate microbial crude protein (Pina et al., 2009). Greater levels of PD are directly related to a more developed rumen.

Final urine volume was increased in heifers fed SB. The SB used in this study was 21% sodium, therefore increasing the sodium consumed by animals on the SB treatment. During the final week of the trial SB heifers consumed on average 121.7 g/d SB therefore consuming 25.6
g/d more sodium than control heifers. Using the equation from Murphy et al. (1983), heifers on the SB treatment would be consuming on average 1,280 mL more water than control heifers. This increase in water consumption would lead to an increase in urine volume.

Apparent total tract digestibility was increased for ADF and OM for animals on the SB treatment. There was a tendency for SB to increase digestibility of DM, CP, NDF, and ash. With the dissociating of SB in the rumen, butyrate is utilized by the rumen to increase growth and concentration of ruminal papillae (Rice et al., 2019; Stahl et al., 2020). This would result in an increase in the surface area of the rumen. The heifer is then more efficient at absorbing nutrients from fermentation. The results of ketone and plasma glucose data seen here support the hypothesis of the SB supplemented animals having a more developed rumen.

Conclusion

Overall, the supplementation of SB to limit fed heifers did not affect growth parameters. Blood ketones and glucose levels were affected indicating development of the rumen due to SB. Coccidia counts were not reduced by SB. Urinary volume was increased on the final week of the experiment for animals supplemented with SB. No effect was seen on purine derivatives. Due to the lack of health benefits or increases in growth parameters observed in the current study there is no justification for the use of SB in limit fed heifer diets.
Table 1. Ingredient composition (% of DM ± SD) of experimental diet

<table>
<thead>
<tr>
<th>Item</th>
<th>DM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Silage</td>
<td>46.17 ± 1.75</td>
</tr>
<tr>
<td>Alfalfa Hay</td>
<td>35.44 ± 2.90</td>
</tr>
<tr>
<td>Energy Mix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.14 ± 0.94</td>
</tr>
<tr>
<td>Soy/Urea Mix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.19 ± 3.55</td>
</tr>
<tr>
<td>Mineral/Vitamin Mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.06 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>1</sup> Energy Mix contains 5% molasses, 45.80% corn meal, 15.20% steam flaked corn, and 34% whole beet pulp

<sup>2</sup> Soy/Urea Mix contains 7.28% distillers grain, 69.14% soy bean meal, 21.83% canola meal, and 1.75% urea

<sup>3</sup> Mineral/Vitamin Mix contains 17.28% Ca; 6.01% P; 3.0% Mg; 23.70% Salt; 7.80% Na; 0.29% Fe; 0.26% Zn; 0.26% Mn; 12.3% Cl; 602.00 mg/kg Cu; 15.00 mg/kg Co; 25.09 mg/kg Se; 15.00 mg/kg I; 267,800 IU/kg Vitamin A; 111,071 IU/kg Vitamin D; and 2,207 IU/kg Vitamin E.
Table 2. Nutrient analysis (% of DM ± SD) of experimental diet

<table>
<thead>
<tr>
<th>Item</th>
<th>DM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>16.92 ± 1.09</td>
</tr>
<tr>
<td>NDF</td>
<td>27.64 ± 2.14</td>
</tr>
<tr>
<td>ADF</td>
<td>38.95 ± 2.50</td>
</tr>
<tr>
<td>Ash</td>
<td>8.50 ± 0.53</td>
</tr>
<tr>
<td>Starch</td>
<td>17.30 ± 2.85</td>
</tr>
<tr>
<td>NFC(^1)</td>
<td>31.53 ± 2.64</td>
</tr>
<tr>
<td>Fat</td>
<td>4.11 ± 0.62</td>
</tr>
<tr>
<td>ME(^2), Mcal</td>
<td>2.27</td>
</tr>
</tbody>
</table>

\(^1\)NFC = 100 – [CP% + (NDF% – NDICP%) + fat% + ash%].

\(^2\)Estimated from NRC (2001).
Table 3. Intake and performance of limit fed heifers fed 0 g/kg sodium butyrate or 0.75 g/kg BW sodium butyrate from 12 to 24 wk of age

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>SB</th>
<th>SEM ³</th>
<th>TRT</th>
<th>TRT X WK ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average BW, kg</td>
<td>135.1</td>
<td>134.5</td>
<td>1.3</td>
<td>0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>ADG, kg/d</td>
<td>0.83</td>
<td>0.86</td>
<td>0.03</td>
<td>0.55</td>
<td>0.008</td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td>99.4</td>
<td>99.7</td>
<td>3.1</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Final BW, kg</td>
<td>168.5</td>
<td>170.4</td>
<td>2.7</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>DMI, kg/d</td>
<td>3.02</td>
<td>2.92</td>
<td>0.044</td>
<td>0.27</td>
<td>0.41</td>
</tr>
<tr>
<td>Feed efficiency, ADG/DMI</td>
<td>0.28</td>
<td>0.29</td>
<td>0.01</td>
<td>0.46</td>
<td>0.009</td>
</tr>
<tr>
<td>Heart girth initial, cm</td>
<td>100.7</td>
<td>103</td>
<td>0.24</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Heart girth, cm</td>
<td>114.7</td>
<td>113.4</td>
<td>0.95</td>
<td>0.37</td>
<td>0.52</td>
</tr>
<tr>
<td>Heart girth gain, cm/d</td>
<td>0.15</td>
<td>0.15</td>
<td>0.006</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>Heart girth final, cm</td>
<td>123.9</td>
<td>123.8</td>
<td>1.1</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Paunch girth initial, cm</td>
<td>121.7</td>
<td>121.7</td>
<td>0.39</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Paunch girth, cm</td>
<td>139</td>
<td>138.2</td>
<td>0.03</td>
<td>0.37</td>
<td>0.65</td>
</tr>
<tr>
<td>Paunch girth gain, cm/d</td>
<td>0.2</td>
<td>0.19</td>
<td>0.009</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Paunch girth final, cm</td>
<td>149.1</td>
<td>148.7</td>
<td>0.07</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Withers height initial, cm</td>
<td>93.5</td>
<td>95.4</td>
<td>0.12</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Withers height, cm</td>
<td>102.8</td>
<td>101.4</td>
<td>0.21</td>
<td>0.06</td>
<td>0.32</td>
</tr>
<tr>
<td>Withers height gain, cm/d</td>
<td>0.1</td>
<td>0.1</td>
<td>0.005</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Withers height final, cm</td>
<td>109.4</td>
<td>108.4</td>
<td>0.63</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Hip height initial, cm</td>
<td>97.6</td>
<td>99</td>
<td>0.17</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Hip height, cm</td>
<td>105.5</td>
<td>105.8</td>
<td>0.4</td>
<td>0.52</td>
<td>0.95</td>
</tr>
<tr>
<td>Hip height gain, cm/d</td>
<td>0.9</td>
<td>0.1</td>
<td>0.004</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Hip height final, cm</td>
<td>111.6</td>
<td>112.3</td>
<td>0.57</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Length initial, cm</td>
<td>81.1</td>
<td>82</td>
<td>0.14</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Length, cm</td>
<td>90.7</td>
<td>90.2</td>
<td>0.58</td>
<td>0.59</td>
<td>0.74</td>
</tr>
<tr>
<td>Length gain, cm/d</td>
<td>0.13</td>
<td>0.11</td>
<td>0.004</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Length final, cm</td>
<td>98</td>
<td>97.4</td>
<td>0.90</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

¹ Treatment CON = 0g/d SB, SB = 0.75 g sodium butyrate/kg BW

² P-value significant if < 0.05; trend if < 0.10

³ Standard error of the mean

⁴ Treatment by week interaction
Table 4. Overall body weight gain and skeletal measurement gains of heifers limit fed 0 g/kg sodium butyrate or 0.75 g/kg BW sodium butyrate from 12 to 24 wk of age

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment 1</th>
<th>P-value 2</th>
<th>SEM 3</th>
<th>TRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, kg</td>
<td>70.4</td>
<td>70.5</td>
<td>2.81</td>
<td>0.99</td>
</tr>
<tr>
<td>Heart girth, cm</td>
<td>21.4</td>
<td>21.4</td>
<td>0.83</td>
<td>0.98</td>
</tr>
<tr>
<td>Paunch, cm</td>
<td>28.4</td>
<td>27.4</td>
<td>1.42</td>
<td>0.64</td>
</tr>
<tr>
<td>Withers, cm</td>
<td>14.1</td>
<td>13.7</td>
<td>0.63</td>
<td>0.99</td>
</tr>
<tr>
<td>Hip height, cm</td>
<td>13</td>
<td>13.7</td>
<td>0.57</td>
<td>0.40</td>
</tr>
<tr>
<td>Length, cm</td>
<td>17.5</td>
<td>16.2</td>
<td>0.82</td>
<td>0.30</td>
</tr>
</tbody>
</table>

1 Treatment CON = 0g/d SB, SB = 0.75 g sodium butyrate/kg BW

2 P-value significant if < 0.05; trend if < 0.10

3 Standard error of the mean
Table 5. Coccidia count and rate, plasma glucose, and whole-blood ketones of heifers limit fed g/kg sodium butyrate or 0.75 g/kg BW sodium butyrate from 12 to 24 wk of age

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>SB</th>
<th>SEM ³</th>
<th>TRT</th>
<th>TRT X WK ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial coccidia/ kg of feces</td>
<td>16.6</td>
<td>15.4</td>
<td>5.8</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Cocci/ kg of feces</td>
<td>8616.5</td>
<td>10815</td>
<td>2991.2</td>
<td>0.61</td>
<td>0.097</td>
</tr>
<tr>
<td>Coccidia rate</td>
<td>0.65</td>
<td>0.56</td>
<td>0.05</td>
<td>0.25</td>
<td>0.19</td>
</tr>
<tr>
<td>Initial glucose, mg/dL</td>
<td>90.9</td>
<td>91.9</td>
<td>0.8</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>95</td>
<td>90.42</td>
<td>1.3</td>
<td>0.03</td>
<td>0.32</td>
</tr>
<tr>
<td>Final glucose, mg/dL</td>
<td>107</td>
<td>92.99</td>
<td>5.2</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td>Initial ketones, mmol/L</td>
<td>0.78</td>
<td>1.2</td>
<td>0.03</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Ketones, mmol/L</td>
<td>0.93</td>
<td>1.55</td>
<td>0.07</td>
<td>&lt;0.0001</td>
<td>0.39</td>
</tr>
<tr>
<td>Final ketones, mmol/L</td>
<td>0.97</td>
<td>1.31</td>
<td>0.07</td>
<td>0.0063</td>
<td></td>
</tr>
</tbody>
</table>

¹ Treatment CON = 0g/d SB, SB = 0.75 g sodium butyrate/kg BW

² P-value significant if < 0.05; trend if < 0.10

³ Standard error of the mean

⁴ Treatment by week interaction
Table 6. Purine derivative excretion of heifers limit-fed 0 g/kg sodium butyrate or 0.75 g/kg BW sodium butyrate from 12 to 24 wk of age

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>SB</th>
<th>SEM 3</th>
<th>TRT</th>
<th>TRT X WK 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary volume, l/d, initial</td>
<td>4</td>
<td>4.5</td>
<td>1.0</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>Urinary volume, l/d, final</td>
<td>9</td>
<td>12.4</td>
<td>0.16</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Urinary volume, l/d</td>
<td>8.9</td>
<td>10.4</td>
<td>0.17</td>
<td>0.34</td>
<td>0.01</td>
</tr>
<tr>
<td>Allantoin, mmol/d, initial</td>
<td>45.5</td>
<td>35.7</td>
<td>5.5</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Allantoin, mmol/d, final</td>
<td>72.9</td>
<td>49.9</td>
<td>21.5</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Allantoin, mmol/d</td>
<td>70.3</td>
<td>43.5</td>
<td>17.2</td>
<td>0.30</td>
<td>0.29</td>
</tr>
<tr>
<td>Creatine, mmol/d, initial</td>
<td>28.5</td>
<td>29.1</td>
<td>0.96</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Creatine, mmol/d, initial</td>
<td>49</td>
<td>49.1</td>
<td>0.74</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>Creatine, mmol/d</td>
<td>44.2</td>
<td>43.6</td>
<td>0.45</td>
<td>0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>Uric acid, mmol/d, initial</td>
<td>12.1</td>
<td>10.8</td>
<td>0.57</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Uric acid, mmol/d, final</td>
<td>18.5</td>
<td>23.6</td>
<td>9.4</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Uric acid, mmol/d</td>
<td>19.4</td>
<td>19.7</td>
<td>6.3</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Total PD, mmol/d, initial</td>
<td>57.6</td>
<td>46.6</td>
<td>5.7</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Total PD, mmol/d, final</td>
<td>99.5</td>
<td>68.2</td>
<td>31.2</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>Total PD, mmol/d</td>
<td>93.9</td>
<td>59.5</td>
<td>23.3</td>
<td>0.32</td>
<td>0.56</td>
</tr>
</tbody>
</table>

1 Treatment CON = 0g/d SB, SB = 0.75 g sodium butyrate/kg BW

2 P-value significant if < 0.05; trend if < 0.10

3 Standard error of the mean

4 Treatment by week interaction
Table 7. Apparent total-tract nutrient digestibility (%), week 6

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>SB</th>
<th>SEM (^3)</th>
<th>TRT</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<tr>
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<td>50.5</td>
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</tr>
<tr>
<td>NDF</td>
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<td>55</td>
<td>1.4</td>
<td>0.06</td>
</tr>
<tr>
<td>HCell(^4)</td>
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<td>66.4</td>
<td>0.15(^5)</td>
<td>0.22</td>
</tr>
<tr>
<td>Ash</td>
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<td>52.8</td>
<td>1.8</td>
<td>0.10</td>
</tr>
<tr>
<td>Starch</td>
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<td>97.7</td>
<td>0.2</td>
<td>0.67</td>
</tr>
<tr>
<td>Fat</td>
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<td>79.2</td>
<td>2.2</td>
<td>0.30</td>
</tr>
<tr>
<td>OM</td>
<td>66.7</td>
<td>71.2</td>
<td>1.4</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(^1\) Treatment CON = 0g/d SB, SB = 0.75 g sodium butyrate/kg BW

\(^2\) P-value significant if < 0.05; trend if < 0.10

\(^3\) Standard error of the mean

\(^4\) Hemicellulose = NDF-ADF

\(^5\) Square root
Chapter III

Effect of sodium butyrate on viability of *Eimeria bovis* sporozoites and LDH release from MDBK cells in the presence of sporozoites

The aim of this study was to determine the viability of sporozoites from *Eimeria bovis* when exposed to sodium butyrate (SB), monensin (MON), or butyric acid (BA), and to determine the effects of SB on sporozoite invasion of cells in comparison to MON. To determine viability, isolated sporozoites were suspended in one of four treatments: control (CON) = RPMI-1640 with 10% FBS, SB = 0.028 mg/mL SB suspended in control medium, MON = 0.01 mg/mL MON suspended in control medium, and BA = 0.18 mg/mL BA suspended in control medium. The number of alive sporozoites were less for the MON and BA treatments than the CON and SB treatments. Monensin and BA were similar in the number of alive sporozoites. There was a trend for treatment to have an effect on sporozoite percent viable based on treatment ($P = 0.1$). Control, SB, and BA treatments were all similar, while MON compared to control and SB had decreased viability. Results for MON when compared to BA were similar. Lactate dehydrogenase release was used to determine cellular damage of Madin Darby Bovine Kidney (MDBK) cells in vitro. These cells were exposed to similar numbers of sporozoites and treated with the following conditions: CON, SB = 0.028 mg/mL SB in control medium, MON = 0.01 mg/mL MON in control medium. Treatment had an effect on LDH absorbance difference. Control LDH absorbance difference was greater than both the SB and MON treatments while the absorbance difference for SB and Mon were similar. Monensin and BA reduced the viability of sporozoites. Sodium butyrate and MON were both shown to decrease cellular damage to MDBK cells as determined by decreased LDH absorbance difference as compared to CON.
Introduction

Coccidiosis is a common disease found on many dairy farms that can cause significant economic losses for producers from the cost of treatment as well as decreased growth rates, veterinary expenses, and reduced feed conversion. It is estimated that the global losses in dairy cattle and bison are $400 million to $700 million (USD) annually (Jolley and Bardsley, 2006). Mortality can also occur with severe cases. Coccidiosis is caused by the protozoan species *Eimeria*. There are many species of *Eimeria* that infect different varieties of livestock. Twelve species have been identified in the feces of cattle. Of the twelve, *E. zuernii*, *E. bovis*, and *E. auburnensis* are most commonly associated with the clinical disease in dairy cattle (Constable, 2015).

From birth, dairy calves are susceptible to infection. Although, symptoms and diagnosis does not occur until 15-21 days after ingestion, due to the parasite’s lifecycle or prepatent period (Jolley and Bardsley, 2006). The route of transmission is fecal to oral. After ingestion of sporulated oocysts from the environment, the life cycle begins. Oocysts are able to survive harsh conditions, making them present in nearly all environments (Jolley and Bardsley, 2006). Symptoms include fever, decreased appetite, weight loss, and dehydration. In the intestine, excysted sporozoites invade intestinal cells becoming merozoites. Merozoites multiply, infecting surrounding cells and differentiate into gametes for reproduction, producing large quantities of oocysts. These oocysts are shed into the environment, allowing for the infection to reach other animals (Pié Orpí, 2020).

With the change of season, temperature fluctuations, and extreme weather events, dairy calves undergo high levels of stress (Matjila and Penzhorn, 2002). This can increase oocyte presence in the environment and make animals more susceptible to infection. Other times of
stress throughout the life of a dairy calf including crowding, shipping, changes in nutrition, and nutritional deficiencies can increase the chance and severity of parasite infection (Jolley and Bardsley, 2006). Coccidiosis due to stress is mediated by increased corticosteroids circulating through the body (Eness, 1985). Subclinical infections are common where animals will appear healthy and the infection will often go undiagnosed, even though oocytes are present in the feces. Although no symptoms are seen, intestinal damage is occurring, causing feed efficiency to be reduced. Chronic infections are seen when bloody diarrhea continuing for more than a week or when thin feces with shreds of epithelium and mucus are seen (Constable, 2015). Enteritis occurs in the large intestine and damage to the mucosa in the small intestine is seen when oocytes are present in the feces (Constable, 2015). The resulting intestinal damage decreases nutrient absorption, hindering the growth of young dairy calves.

Ionophores, a polyether antibiotic, were introduced to the broiler industry for the prevention and control of coccidiosis known commonly as coccidiostats (McGuffey, 2017). In the 1970’s, the dairy industry began the use of ionophores. Monensin (MON) is commonly used on US dairy farms today for coccidiosis control as a feed additive to promote growth and reduce coccidiosis. The antibiotic effect of MON is due to its mechanism of action, by which it affects Gram positive bacteria causing a reduction and thereby enhancing Gram negative bacteria and the production of propionic acid. Monensin causes a large uptake of Na$^+$ ions into the cell and the inhibition of the Na$^+$-K$^+$-ATPase pump (Smith and Galloway, 1983). This increases intracellular osmotic pressure and causes water to flow in and result in cellular lysis. The use of ionophores will not prevent infection or eradicate the parasite, rather will reduce infections to subclinical levels. Coccidiostats are widely used in the poultry industry, some species of *Eimeria* have developed resistance to certain coccidiostats (Jolley and Bardsley, 2006). Recently, more
pressure has come from consumers to reduce antibiotic usage due to the potential development of antibiotic resistance. The European Union banned the use of ionophores in livestock feeds (European Commission, 2005). This has led researchers to seek alternative feed additives that can ameliorate the effects of coccidiosis.

Recent studies with sodium butyrate (SB) have been shown to decrease coccidial infections (Rice et al., 2019; Stahl et al., 2020). Heifers starting at 12 weeks of age were supplemented with differing concentrations (0, 0.25, 0.50, 0.75g/ kg of body weight(BW)) of SB and were seen to have an increase in BW ($P = 0.04$), a tendency for greater feed efficiency ($P = 0.08$) and final BW ($P = 0.07$), and a reduction of coccidial oocysts in feces ($P = 0.03$; Rice et al. 2019). Stahl et al., (2020) supplemented diets with 0.75g/ kg BW SB, MON (1 mg/kg BW), or a combination of SB and MON, to evaluate if SB fed heifers responded similarly to MON fed heifers. When fed either additive or the combination, animals tended to have greater average body weight ($P = 0.10$), increased dry matter intake ($P = 0.03$), and a reduction in the prevalence of coccidian oocysts ($P = 0.03$) compared to animals not fed an additive (Stahl et al., 2020). The rate of diarrhea has also been shown to be decreased with the supplementation of different forms of SB. Wu et al., 2022, supplemented free or fat coated SB to calves in starter grain and observed the diarrhea rate to drop in the free SB and rapid release SB treatments ($P < 0.05$). Diarrhea is commonly linked to a coccidia infection.

To date, no in vitro experiments have been performed to investigate the effects of SB on coccidia sporozoites. The objective of this study was to determine the viability of sporozoites in vitro when SB, MON, or butyric acid (BA) was present and to determine the effects of SB on sporozoite damage to bovine epithelial cells in comparison to MON. It was hypothesized that SB would reduce viability of sporozoites and decrease cell damage.
Materials and Methods

Cell Culture and Reagents

Mardin Darby Bovine Kidney (MDBK) cells were obtained from the American Type Culture collection (ATCC CCL-22, Manassas, VA). The cell line was cultured in Roswell Park Memorial Institute 1640 (RPMI 1640); (Sigma-Aldrich, St. Louis, MO) medium with 10% Fetal Bovine Serum (FBS) in a humidified incubator at 37°C with a 5% CO₂ atmosphere. Treatments (SB, MON, and BA) were purchased from Sigma-Aldrich. Treatments were dissolved in differing concentrations in RMPI-1640 with 10% FBS. A concentrated stock solution of MON was first dissolved in 100% ethanol as an intermediate solvent due to its low solubility in water.

For each treatment a dose-response was measured to determine the dose of treatment. All selected concentrations were seen to be effective without cellular harm. Concentrations from 0 mg/mL to 0.898 mg/mL SB were tested with 0.028 mg/mL SB as the chosen concentration. Monensin concentrations ranged from 0 mg/mL to 0.08 mg/mL with 0.01 mg/mL MON as the chosen concentration. Concentrations from 0 mg/mL to 1.42 mg/ml BA were tested with 0.18 mg/mL BA as the selected concentration.

Coccidia Oocyst Isolation

Fecal samples were obtained from the Fairchild Dairy Teaching and Research Center at the University of New Hampshire in Durham, NH. The modified Wisconsin sugar fecal worm egg flotation method was performed on fecal samples to determine oocysts/gram of feces (Bliss and Kvasnicka, 1997). Fecal samples were then diluted with tap water and passed through a
series of metal sieves with pore sizes of 850, 400, 149, 88, and 49 micrometers to separate oocysts from debris. The diluted and filtered sample was mixed 1:1 volumetrically with a saturated sucrose solution. The saturated sucrose solution was made by dissolving 454 g sucrose in 355 mL deionized water with constant stirring on a hot plate and was cooled to room temperature before use. The diluted sample was then transferred to a 23 × 33 cm shallow glass dish and covered with a plastic sheet that was in contact with the solution. Every 2-10 h the plastic sheet was washed with a squirt bottle filled with deionized water into a large beaker. This was performed for 48 h with washing happening every 2 h for the first 12 h and then every 9 h thereafter, the wash was centrifuged at 600 × g for 12 min to collect oocytes. The remaining pellet was resuspended in 2% potassium dichromate in water. The suspension was covered and placed into a water bath at 28 °C under constant aeration for 48 h. After, the suspension was centrifuged at 600 × g for 12 min and resuspended in 2% potassium dichromate and stored at 4°C for future use.

**Oocyst Excystation**

Oocyst samples stored in 2% potassium dichromate were centrifuged at 2,000 × g for 10 min. The supernatant was aspirated off. Samples were then washed in 1% sodium hypochlorite on ice for 20 min, centrifuged at 2,000 × g for 10 min and the supernatant was removed. Samples were further washed 5 times by repeated centrifugation for 10 min at 2,000 × g in sterile deionized water. Oocysts were then suspended in 0.02 M L-cysteine HCl-H2O (Sigma-Aldrich) /0.2 M NaHCO₃ (Sigma-Aldrich) solution and incubated for 20 h at 37°C in a 100% CO₂ atmosphere in a vented T75 cell culture flask. To remove the solution, the sample was centrifuged for 10 min at 2,000 × g and the supernatant was removed. Samples were suspended
in excystation solution (Hanks Balanced Salt Solution (HBSS) with 0.4% trypsin (Sigma-Aldrich) and 8% bovine bile (Sigma-Aldrich)) and incubated for 2 h at 37°C in a 5% CO₂ atmosphere in a vented T75 cell culture flask. The sample was then washed until clear with sterile phosphate-buffered saline (PBS) by centrifugation at 2,000 × g for 10 min. Samples were suspended in Minimum Essential Medium (MEM). Due to the presence of bacterial contamination, samples were suspended in a gentamicin solution (100 μg/mL (Schafer et al., 1971);(Sigma-Aldrich) for one hour inside a vented T75 cell culture flask at 37°C in a 5% CO₂ atmosphere. Sterile sporozoites were then suspended in RPMI 1640 with 10% FBS. Sterility tests performed for contamination were negative.

**Sporozoite Viability**

Sporozoites were suspended in different sterile treatments and control to determine their effects on sporozoite viability. A 24-well sterile cell culture plate was used with each well containing 0.25 mL CON medium with sporozoites suspended in solution. Treatments were replicated in 4 wells for each treatment. Treatments were as follows: CON, SB (0.028 mg/mL), MON (0.01 mg/mL), and BA (0.18 mg/mL). Each well received 1 mL of treatment medium with 0.25 mL medium with sporozoites suspended in solution resulting in a total of 1.25 mL per well. The multiwell plate was incubated at 37°C in a 5% CO₂ atmosphere for 48 h. After incubation, a dye exclusion test using trypan blue was used to determine sporozoite viability (Nakai and Ogimoto 1983; Khalafalla et al. 2011). Trypan blue is a vital dye that is taken up by dead or dying cells that have cell membrane damage, 0.1 mL trypan blue (0.4%) was added to 0.8 mL of medium from each individual well (n=4). Dead or dying cells appear blue, having taken up the dye, while live cells appeared a light golden color. Glass slides were prepared by pipetting the
sporozoite containing medium with dye onto a glass slide and covering with a coverslip. Sporozoites were visualized using bright field microscopy at 200 x magnification to distinguish living and dead sporozoites. Counts were performed to determine percent viable sporozoites. Five random sections on each slide were counted by two different individuals and recorded. The number of alive and dead sporozoites were recorded. To calculate percent viable, the number of live sporozoites was divided by the total number of sporozoites, dead and alive.

**Lactate Dehydrogenase (LDH) Assay**

A LDH assay was used to investigate potential cellular damage by sporozoites to normal healthy mammalian epithelial cells in vitro. Madin Darby Bovine Kidney cells were seeded into a 24-well plate at 50,000 cells per well and grown to approximately 85% confluency. To each well, 0.25 mL CON medium was added with sporozoites suspended in solution. Treatments were assigned to four wells within the column on the plate (n=4). Treatments were as follows, SB (0.028 mg/mL), MON (0.01 mg/mL), sporozoites, and CON containing no sporozoites. To each well 1.25 mL of the given treatment was added, and the plate was incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. The medium from each well was placed into separate labeled microcentrifuge tubes and centrifuged for 5 min at 1500 × g to ensure no cells were present in the medium that was assayed for LDH release. The supernatants from each tube were then stored frozen at -20°C until analysis.

Samples were thawed at room temperature. An LDH assay kit (CyQUANT™ LDH Cytotoxicity Assay, Thermofisher Scientific, Waltham MA) was used to determine levels of LDH in each well. Each sample was assayed in duplicate. Samples were analyzed
colorimetrically using a microplate reader (Epoch Bio Tek Instruments, Inc., Winooski, VT) set at wavelengths of 490 and 680 nm for two readings of each sample. Absorbance values were then calculated by subtracting the 680 nm absorbance value from the 490 nm absorbance value to remove background signal from the instrument as instructed in the assay kit.

**Statistical Analysis**

Sporozoite viability including total, dead, alive, and percent viability were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The following model was used: \( Y_{ijk} = \mu + \text{Trt}_i + S_j + C_k + E_{ijk} \) where \( Y_{ijk} \) = the dependent variable; \( \mu \) = the overall mean; \( \text{Trt}_i \) = the fixed effect of the \( i \)th treatment (\( i = \) control, SB, MON, BA); \( S_j \) = the fixed effect of the \( j \)th section (\( k = 1-5 \)); \( C_k \) = the fixed effect of the \( k \)th counter (\( c = 1, 2 \)); and \( E_{ijk} \) = the residual error \( \sim N(0, \sigma^2_e) \). Least square means were separated using the P.DIFF function of SAS 9.4.

The LDH assay results were analyzed using MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with the following model: \( Y_i = \mu + \text{Trt}_i + E_i \) where \( Y_i \) = the dependent variable; \( \mu \) = the overall mean; \( \text{Trt}_i \) = the fixed effect of the \( i \)th treatment (\( i = \) control, SB, MON, sporozoites); \( E_i \) = the residual error \( \sim N(0, \sigma^2_e) \). Least square means were separated using the P.DIFF function of SAS 9.4.

Treatment effects were deemed significant when \( P \leq 0.05 \) and trends at \( 0.05 < P \leq 0.10 \). All data points greater or lesser than 3 SD away from the mean were considered outliers and removed from the dataset.
Results

Concentrations of treatments of SB, MON and BA were used that would allow for MDBK cell survival in culture and still be effective.

Sporozoite viability and LDH assay results are presented in Table 8. No differences were observed between treatments for total sporozoites per well. There were numerical differences in the total number of sporozoites per well. Treatment also had no effect on the percentage of dead or dying sporozoites as indicated by trypan blue. However, MON or BA, had an effect to decrease live sporozoites counted ($P = 0.009$). There was no difference between in alive sporozoites counted for CON and SB or MON and BA treatments. There was a difference between control and MON ($P = 0.006$), control and BA ($P = 0.01$), SB and MON ($P = 0.02$), and SB and BA ($P = 0.04$) for percent live sporozoites. A trend was observed for treatment to decrease sporozoite percent viability ($P = 0.1$). Control, SB, and BA treatments were all similar while MON compared to CON ($P = 0.03$) and SB ($P = 0.04$) were different. Results for MON when compared to BA were similar.

Results for the degree of potential damage to MDBK cells by sporozoites in vitro using a LDH assay are presented in Table 8 and Figure 2. Treatment had a significant effect ($P < 0.001$) on LDH levels. Lower absorbance values indicate less cell membrane damage. The control treatment LDH release was the similar to both the SB ($P = 0.20$) and MON treatments ($P = 0.55$). While the sporozoite treatment was greater than the SB ($P < 0.001$) and MON treatments ($P < 0.001$). Cells constitutively release LDH, with additional LDH occurring during cellular damage. The sporozoite treatment had the highest LDH release.
Discussion

*In vitro*, MON was shown to affect the sporozoite life stage of *Eimeria tenella*. This was due to the large uptake of Na$^+$ ions and the inhibition of Na$^+$ ions out of the cell through the Na$^+$-K$^+$-ATPase pump (Smith and Galloway, 1983). This increases intracellular osmotic pressure, causing water to flow in and result in cellular lysis. Previous research showed that MON causes merozoites to swell and burst resulting in sporozoites being released and coming into contact with the antibiotic (Mehlhorn et al., 1983). Thus, the MON treatment produced a lower percent of living sporozoites.

Sodium butyrate is composed of Na$^+$ ionically bound to butyric acid. In solution, the Na$^+$ has the potential to dissociate from the butyric acid. This may explain why both the MON and BA treatments caused a decline in the percent viability in sporozoites. Butyric acid and its derivatives have been used in research associated with colon cancer in humans. Pattayil et al. (2019) preformed a study looking at the morphological assessment of apoptotic cells with the inclusion of butyrate derivatives. One of the derivatives used here was sodium butyrate. Researchers found that when human colorectal carcinoma cells (HCT116) were treated with butyric acid derivatives, it induced apoptosis (Pattayil et al., 2019). Sodium butyrate has been supplemented to milk replacer and starter grain of preweaned calves (Górka et al. 2011; 2014). Researchers observed an increase in mitotic indices and a decrease in apoptotic indices of small intestine enterocytes suggesting an enhanced maturation of mucosal cells. In chickens, *Eimeria* alters the gut microbiota, more importantly short chain fatty acids reducing their prevalence, including butyrate (Leung et al., 2018). Butyrate is known to reduce inflammation in the gut (Andoh et al., 1999; Segain et al., 2000; Song et al., 2006; Elce et al., 2017). Therefore, increasing levels of butyric acid in the gut can help to reduce inflammation and the severity of
coccidiosis. Our data support the *in vivo* observations of reduced oocyst shedding in heifers fed SB (Rice et al., 2019; Stahl et al., 2020). Zhou et al., (2017) showed that microencapsulated SB decreased the abundance of *Bacteroides* abundance in *E. tenella* infected chickens indicating that SB can alter the microflora of the intestine of infected birds. Bortoluzzi et al. (2018) observed that SB modulated the diversity of the microbiota present in the intestinal tract of chickens challenged with *E. maxima* reducing the negative impact of necrotic enteritis. Using tributyrin, Wang et al., (2021) observed that broiler chicks exposed to a mix of *Eimeria* and fed tributyrin had greater villus heights and widths and reduced oocyst shedding between d 19-26 (*P* < 0.05). However, it is not clear from these studies if the response was due to anticoccidial effects of SB or the effect of SB on the intestinal epithelium or both.

Lactate dehydrogenase is an important enzyme of the anaerobic metabolic pathway and is found within the cytoplasm of many cells. It is constitutively released from cells in low quantities, however, release above normal control values are indicative of cell membrane damage. The control treatment acted as the “normal” levels of LDH that would be released from MDBK cells. The function of the enzyme is to catalyze the reversible conversion of lactate to pyruvate with the reduction of NAD+ to NADH and vice versa.

Results from the LDH assay are supported by results from Rice et al. (2019) and Stahl et al. (2020) when SB was supplemented *in vivo* to heifers. Rice et al. (2019) supplemented varying amounts of SB, 0, 0.25, 0.5, and 0.75 g/kg of body weight to the diet of a heifer. These researchers saw a quadratic effect on SB reducing the prevalence of coccidia in the feces. Stahl et al. (2020) supplemented heifer diets with SB, MON, or a combination (SB+MON). A decrease in coccidia in the feces was seen when animals were supplemented with an additive (SB, MON, or the combination). Their research supports the present findings on LDH absorbance difference
in the SB and MON treatments being lower than the sporozoite treatment constitutive release. Dead or dying sporozoites are not viable to invade and damage the MDBK cells, thus decreasing cellular damage and quantity of LDH released. As described above, MON causes sporozoites to lyse protecting the host cell from sporozoite invasion and subsequent damage. However, one in vivo study supplementing SB did not see a reduction in coccidia in heifers supplemented with SB (Klobucher, 2022). Heifers were limit fed in this study, this decreases the rate of passage of feed through the gut. Feed then ends up staying in the upper gastrointestinal tract longer than it does for ad libitum fed animals (Zanton and Heinrichs, 2008; Pino et al., 2018). This allows for animals to absorb more nutrients out of the feed therefore, improving feed efficiency (Pino et al., 2018). In the study reported here, it was assumed that the SB dissociated in the rumen, and the butyric acid was absorbed in the upper gastrointestinal tract. This is supported by the fact that when animals are supplemented with SB, they have higher level of ketones circulating in the blood during the 12 wk trial and in their final measurements compared to the control animals. Overall, in this study, no difference was seen in average coccidia/ kg feces and incidence rate between the control and SB animals due to SB being absorbed in the upper gastrointestinal tract and therefore not reaching the small intestine (Klobucher, 2022).

Conclusion

Butyric acid had similar anticoccidial activity as MON on the number of live sporozoites compared to SB and control. Sodium butyrate and MON caused a decrease in LDH compared to the sporozoite treatment indicating that fewer sporozoites in these wells damaged the MDBK cells potentially due to lower viability of sporozoites in these treatments. Further research is
needed to see the exact mechanism of how BA and SB affect sporozoites both in vitro and in vivo.
Table 8. Sporozoite viability and MDBK cell damage based on lactate dehydrogenase release

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>SB</th>
<th>MON</th>
<th>BA</th>
<th>Sporozoites</th>
<th>SEM</th>
<th>TRT</th>
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<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>13.6</td>
<td>11.8</td>
<td>11.9</td>
<td>-</td>
<td>1.1</td>
<td>0.38</td>
</tr>
<tr>
<td>Dead</td>
<td>5</td>
<td>5.3</td>
<td>6.7</td>
<td>6.4</td>
<td>-</td>
<td>0.8</td>
<td>0.36</td>
</tr>
<tr>
<td>Alive</td>
<td>9 (^a)</td>
<td>8.3 (^a)</td>
<td>5.1 (^b)</td>
<td>5.6 (^b)</td>
<td>-</td>
<td>0.98</td>
<td>0.009</td>
</tr>
<tr>
<td>Percent viable</td>
<td>62.5 (^a)</td>
<td>61.2 (^a)</td>
<td>44.3 (^b)</td>
<td>51.3 (^a),(^b)</td>
<td>-</td>
<td>5.8</td>
<td>0.10</td>
</tr>
<tr>
<td>LDH, abs(^5)</td>
<td>0.23 (^a)</td>
<td>0.26 (^a)</td>
<td>0.24 (^a)</td>
<td>-</td>
<td>0.43 (^b)</td>
<td>0.24</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^a,b\) Denotes differences in least mean squares (\(P = <0.05\))

1 Treatment CON = RPMI-1640 with 10% FBS, SB = 0.028 mg/mL sodium butyrate, MON = 0.01 mg/mL monensin, BA = 0.18 mg/mL butyric acid, Sporozoites = RPMI-1640 with 10% FBS w/sporozoites

2 P-value significant if < 0.05; trend if < 0.10

3 Sporozoites = sporozoites and MDBK cells

4 Standard error of the mean

5 LDH = Lactate dehydrogenase absorbance difference (680nm – 490nm). Data were transformed by taking the inverse to allow normality. Data presented were transformed back after statistical analysis. The standard errors were not transformed back.
**Figure 2:** Lactate Dehydrogenase Release from MDBK cells

![Graph showing Lactate Dehydrogenase Release Assay](image)

\[ \text{LDH} = \text{Lactate dehydrogenase absorbance difference (680nm – 490nm)} \]

- \( \text{LDH}_{\text{abs}} \)

1. **Treatment**
   - CON = RPMI-1640 with 10% FBS
   - Sporozoites = RPMI-1640 with 10% FBS with sporozoites
   - SB = 0.028 mg/mL sodium butyrate with sporozoites
   - MON = 0.01 mg/mL monensin with sporozoites
   - BA = 0.18 mg/mL butyric acid with sporozoites

2. Denotes differences in least mean squares \( (P < 0.05) \)

Data were transformed by taking the inverse to allow normality. Data presented were transformed back after statistical analysis. The standard errors were not transformed back.
List of References:


Development, Nutrient Digestibilities, and Nitrogen Utilization in Holstein Calves.”


26-Feb-2021

Erickson, Peter S
Agriculture, Nutrition, & Food Systems
Keener Dairy Research Building
Durham, NH 03824

IACUC #: 210201
Project: Supplementing Sodium Butyrate to Limit-fed Heifers: Effects on Growth, Health, Nutrient Digestibility, and In Vitro Anti-coccidial Activity
Approval Date: 25-Feb-2021

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category C in Section V of the Application for Review of Vertebrate Animal Use in Research or Instruction - Animal use activities that involve normal maintenance, or breeding, conditioning, or holding (with IACUC approval) for future use in teaching, testing, experiments, research or surgery. The IACUC made the following comment(s) on this protocol:

1. The IACUC administrator made the following changes to the protocol:
   1. In Section IV, C, #3, changed bi-weekly to weekly for the blood draw.
   2. Added 10 Jersey Heifers, USDA category C, to Section V, Table 1.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike.

Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Susan Jalbert at 862-3536.

For the IACUC,

Julie Simpson, Ph.D.
Director

cc: File