EVALUATING THE IMPACT OF BOVAMINE® ON PERFORMANCE, NUTRIENT DIGESTIBILITY, AND DIGESTIVE FUNCTION IN LACTATING DAIRY COWS

by

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ABSTRACT

Two experiments evaluated the effect of a direct fed microbial (DFM) (Bovamine®, Chr. Hansen, Hørsholm, Denmark) on performance and digestion of dairy cows. In Experiment 1 (Exp1), 30 multiparous cows (75 ± 32 DIM) were assigned to one of two treatments fed for 10 weeks, BOV (3 x 10^9 CFU/d Bovamine®) or CON (control, no Bovamine®). In Experiment 2 (Exp2), 6 ruminally cannulated cows (123 ± 129 DIM) were assigned to a crossover design with two 6 week periods with the same treatments as Exp1 except that cows were fed a 23.8% starch diet during weeks 1 – 5 of each period then abruptly switched to a 31.1% starch diet for week 6. For both experiments, intake and milk yield were measured daily and milk samples were collected weekly. In Exp1, fecal grab samples were collected every 6 h on d 7 of week -1, 1, 2, 4, 6, 8, and 10, fecal consistency was scored, and fecal starch measured in composited daily samples. Composites from a subset of 7 cows per treatment were used to measure apparent total tract nutrient digestibility. In Exp2, rumen pH was continuously recorded during weeks 5 – 6. Rumen in situ digestibility was measured on week 5 day 7, week 6 day 1, and week 6 day 7. On those dates, rumen fluid and feces were collected every 6 h for rumen VFA, fecal pH, and fecal starch (composited by cow within day). Rumen and fecal microbiome samples were collected at one time point on these days. In Exp1, treatment did not affect intake, milk yield or composition, fecal score or fecal starch. BOV tended to increase starch digestibility compared to CON (98.74 vs. 98.46%, $P = 0.051$), but digestibility of other nutrients was unaffected. In

Exp2, intake, milk yield, and milk composition were unaffected when evaluated over the entire study. However, during the abrupt switch to the high starch diet, milk fat yield was increased for BOV compared to CON (1.39 vs. 1.28 kg/d, $P = 0.002$) and milk fat percentage tended to increase (3.59 vs. 3.42%, $P = 0.09$). Treatment did not impact rumen pH, rumen VFA, in situ digestibility or the rumen and fecal microbiomes. Contrary to Exp1, BOV increased fecal starch compared to CON (2.49 vs. 2.03%, $P = 0.02$), and this was most evident during the high starch feeding. Overall, Bovamine® modestly improved starch digestibility in Exp1 and increased milk fat during the high starch challenge in Exp2.

Chapter 1

LITERATURE REVIEW

1.1 Dairy Cow Efficiency

In today's market, dairy farmers are struggling with exceptionally low milk prices. Many producers are selling out, going out of business, or just breaking even. The USDA forecasts the price of milk for 2018 to be $16.20-$16.70 per hundredweight (cwt). In order for a dairy farm to be profitable, their annual expenses need to remain lower than their income. In most operations, the cost of feed is a producer's largest cost of producing milk. But if farmers are able to raise cows that can utilize feed more efficiently, they can increase their profit margin. This means cows will put less feed towards maintenance, and more towards production resulting in a higher volume of milk per unit of feed. Being able to make cows more efficient will help lower the cost of production of milk. In addition, by making the dairy cow more efficient she will also be able to better utilize nutrients. Better utilization will result in a decrease of nutrients, like nitrogen and phosphorus, back into the environment.

There are multiple strategies producers use to raise more efficient dairy cows. Recent work by geneticists focuses on breeding cows that are more efficient by requiring less food to produce the same volume of milk. This can be possible by identifying genetic markers for feed efficiency (VandeHaar, 2014). Being able to breed feed efficient cows will allow producers to reduce feed costs without having an impact on production. Another way to improve feed efficiency in dairy cows is to provide a

ration that is tailored for their stage of production. Throughout lactation dairy cows have different nutritional needs to meet energy requirements. If producers can better meet their specific requirements by grouping and feeding cows based upon where they are in lactation, efficiency can be maximized. Forage quality is also an important factor. The higher the quality of forage, the more digestible it is for the dairy cow, which leads to increased intake and milk production. The amount of energy cows spend exercising can also influence feed efficiency. If cows are required to walk far distances to and from the parlor, and to the waterer or feed bunk, they are expending too much energy on maintenance and not towards production (Hutjens, 2012). Providing proper barns and facilities to limit exercise and maximize comfort can help increase profitability. As illustrated above, there are a variety of genetic, nutritional, and management options available to help a producer improve dairy herd efficiency. Another nutritional option and the focus of this thesis is supplementation of dairy cattle rations with direct fed microbials (DFM).

1.2 Direct Fed Microbials (DFM)

Probiotics in both animal and human food systems are defined as "a live microbial feed supplement that may beneficially affect the host animal upon ingestion by improving its intestinal microbial balance" (Fuller, 1989). A DFM has a broader definition defined by the United States Food and Drug Administration as "a source of live, naturally occurring microorganisms." The concoction of the live organism cultures used in DFMs should beneficially affect the host. The idea is to establish a healthy and

desirable gut microflora, and to prevent the colonization of enteric pathogens. Microorganisms used as DFMs in ruminants consist of viable fungi and bacterial cultures. These DFMs target the rumen where they must be able to actively survive the environment. For this reason, research is limited to a few genera. Feeding DFMs to ruminants can potentially decrease methane production, reduce feed protein degradation, and improve carbohydrate fermentation and fiber digestibility (Jouany and Morgavi, 2007; Chaucheyras-Durand and Durand, 2010).

While certain yeasts and aerobic fungi can be found within the rumen, they are usually nonfunctional and found in small numbers (Brown and Nagaraja, 2009). Despite this, benefits have been observed from supplementing cattle diets include yeasts and molds, like *Saccharomyces cerevisiae* and *Aspergillus niger* respectively. Feeding fungal DFM increases the concentration of rumen bacteria and aids in the stimulation of fiber digesting and lactate utilizing bacteria. It is still unknown how yeasts are able to stimulate microbial growth, but it is theorized that they provide micronutrients to the indigenous rumen microbes (Brown and Nagaraja, 2009). They have also been shown to increase propionic acid and decrease lactic acid concentrations (Newbold et al., 1995). When the production of lactic acid increases, ruminal pH drops. At low pH other bacteria in the rumen environment experience suppressed growth (Russell & Hino, 1985). Excessive accumulation of lactic acid also leads to deceased appetite, sickness, and in severe cases death of the animal. Therefore, decreasing lactic acid concentration makes for a more favorable rumen environment.

Bacterial DFMs are frequently composed of lactic acid producing bacteria and/or lactic acid utilizing bacteria. These usually include *Lactobacillus, Propionibacterium, Bacillus, Bifidobacterium,* and *Enterococcus* species (Elghandour et al., 2015). Some DFMs have been shown to be beneficial by increasing daily gain and feed efficiency in feedlot cattle, enhancing milk production in dairy cows, and improve health and performance in young calves (Uyeno et al., 2015).

Ruminal Effects

The price dairy farmers receive for their milk is based upon the amount of fat and protein that comprise the milk that they are producing. These components determine the value of the milk. DFMs may have an economic benefit by enhancing ruminal fermentation to have an effect on milk composition. Milk protein is synthesized in the mammary gland from amino acids and makes up 3-4% of the milk composition. Therefore, an adequate amount of amino acids should be supplied to the dairy cow (Rehberger et al., 2008). During intestinal digestion dietary proteins are broken down to amino acids that are absorbed into the body. Rumen microorganisms are also able to degrade carbohydrates provided by the diet into volatile fatty acids (VFAs) which provide energy for the cow (Uyeno et al., 2015). Ruminal VFAs affect fat and protein concentrations in milk. For example, increasing propionate production increases protein concentration, and increasing acetate production increases fat concentration (Rehberger et al., 2008). High producing, early lactation, dairy cows are typically in a negative energy balance where they are unable to consume enough feed to meet their energy

requirements. Energy balance can be positive or negative and is defined as the difference between the amount of energy that is consumed by the animal and the amount of energy which that animal expends. Providing a proper diet to meet the dairy cow's energy requirements is essential to maintain a healthy and functional rumen which is directly related to the production and the profit of the dairy farmer.

When including DFMs into dairy cow rations it is important to consider the already present microbial population of the host. This indigenous population has already adapted to the gastrointestinal tract (GIT) environment in a symbiotic relationship between themselves and the animal, creating a delicate balance that if disturbed can cause illness to the host. An example is subacute ruminal acidosis (SARA) which is a common health problem in cattle caused by dysfunction within the rumen. This occurs when there is an accumulation of VFAs in the rumen and pH falls below 5.8 (Kleen et al., 2003). Khafipour et al. (2011) reported that dairy cows under SARA conditions harbored increased numbers of virulence *Escherichia coli* in the rumen, which leads to illness of the animal. Another important consideration when choosing to feed a DFM is any ingested microbes provided need to be able to adapt to the rumen environment. The microorganisms have to be able to inhabit a suitable niche in order to exert beneficial effects on the health of the host. In cattle these niches may include the rumen epithelium, rumen fluid, or on fibrous feedstuffs (Uyeno et al., 2015).

In order for a bacterial DFM to be effective the bacteria being fed need to meet several key criteria. The strain needs to be safe, survive the gut environment, be specific to the host, and show genetic stability (Holzapfel et al., 1998). The mode of action

which DFMs work is still debated by researchers. Mechanisms depend on the product that is fed, and the amount and frequency of feeding (Elghandour et al., 2015). In cattle, there is little work on investigating the mode of action of bacterial DFM and their ability to colonize the rumen and/or intestines.

Seo et al. (2010) propose several different modes of action for DFMs in the rumen. Four common mechanisms for lactic acid producing bacteria include: providing a constant lactic acid supply, adaptation of the microflora to the accumulation of lactic acid, stimulate lactate utilizing bacteria, and the stabilization of ruminal pH. Lactic acid utilizing bacteria have five modes of action: conversion of lactate to VFA, production of propionic acid, increased feed efficiency, decreased methane production, and increased ruminal pH. Lactic acid producing bacteria are able to provide a steady and low concentration of lactate in the rumen which constantly stimulates lactic acid utilizing bacteria. The lactic acid utilizing bacteria prevent the accumulation of lactate in the rumen reducing the risk for acidosis (Nocek et al., 2002).

Intestinal Effects

Most studies on feeding bacterial DFMs to ruminants primarily focus on their effects in the rumen and different fermentation parameters. But there may be some beneficial effects that DFMs have on the post ruminal GIT. Some groups state that in general, bacterial DFM should have more of an impact in the lower gut, and fungal DFM more in the rumen (Brown and Nagaraja, 2009). Seo et al. (2010) propose the

following modes of action which bacterial DFM have the potential to be beneficial in the intestines of ruminants.

(1) Produce antibacterial compounds such as acids, bacteriocins and antibiotics. Lactic acid bacteria can produce hydrogen peroxide that can oxidize bacterial cells (Dicks and Botes, 2010), blocking glycolysis (Carlsson et al., 1983), which has been shown to inhibit *Staphylococcus aureus* and *Pseudomonas* species (Holzapfel et al., 1995). Silva et al. (1987) has also shown that a *Lactobacillus* isolated from humans was able to produce antimicrobial compounds that reduced the growth of *Staphylococcus, Streptococcus,* and *Pseudomonas* species *in vitro. (2) Compete with pathogens for nutrients and/or colonization sites in the mucosa.* Bacterial DFM can outcompete pathogens for adherence sites in the gut to help prevent illness of the host. Attachment of beneficial bacteria in a specific niche also allows proliferation of the organism. Lactic acid producing bacteria have been shown to protect mice from the colonization of *Salmonella* by adhering to the intestinal tract (Frizzo et al., 2010). Lee et al. (2003) reported that supplementing *Lactobacillus* to humans limited the ability of pathogens to attach to intestinal epithelial cells. *(3) Produce or stimulate the production of enzymes.* Jejunal enterocytes can increase enzyme activity and production with the inclusion of a DFM (Chichlowski et al., 2007). *(4) Stimulate an immune response by the host.* When DFMs are administered to the GIT they are taken up by intestinal epithelial cells by transcytosis. They are engulfed by antigen presenting cells, macrophages or dendritic cells which stimulates an immune response (Dicks and Botes, 2010). Some lactic acid producing bacteria strains have been shown to initiate an immune response

by activating macrophages to produce cytokines (Miettinen et al., 1996; Matsuguchi et al., 2003). Previous work feeding DFM to ruminants has primarily focused on their effects in the rumen and not in the intestines. But studies in poultry, mice, and humans suggest that DFM may have beneficial effects in the intestines of ruminants if these products are able to survive and make it through the rumen environment.

1.3 *Lactobacillus acidophilus* & *Propionibacterium freudenreichii*

Lactobacillus acidophilus is a gram-positive rod-shaped facultative anaerobe and is homofermentive (Axelsson, 2004). The only byproduct it forms from fermentation is lactic acid (Kullen and Klaenhammer, 1999). During glycolysis, pyruvate is reduced to lactic acid by NAD^+ dependent lactate dehydrogenase (Axelsson, 2004). The rumen is a perfect environment for *L. acidophilus* to thrive since these bacteria prefer optimal temperatures from 37 – 42ºC and a pH of 5.5 – 6.0 (Altermann et al., 2005). Lactic acid production and utilization within the rumen positively correlates with feed efficiency and animal health (Seo et al., 2010). For example, it has been shown that *L. acidophilus* may decrease the risk of SARA. In a study by Huffman et al. (1992) *L. acidophilus* reduced the amount of time that ruminal pH was below 6.0 in steers switched to a high concentrate diet. It is also suggested that *L. acidophilus* causes other microorganisms in the rumen to adapt to the presence of lactic acid (Krehbiel et al., 2003). If lactic acid utilizing bacteria are constantly being stimulated, the total lactic acid available in the rumen and total ruminal acidity would decrease (Nocek et al., 2000). In turn, pH would remain stable creating an environment for

optimal feed efficiency. The concept of feeding a lactic acid producing DFM to cattle in order to maintain that constant lactic acid supply is difficult to believe when the rumen is already colonized with many lactic acid producing bacteria. But there is evidence to support the reduced risk of ruminal acidosis when feeding lactic acid producing bacteria (Ghorbani et al., 2002; Nocek and Kautz, 2006; Oetzel et al., 2007). *Lactobacillus* species have also shown to have an inhibitory effect against pathogens that may be in the rumen by producing antimicrobial bacterial proteins (Krehbiel et al., 2003). *L. acidophilus* demonstrates the ability to be antagonistic towards pathogenic *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and *Clostridium perfringes,* likely by decreasing pH (Gilliland and Speck, 1977). *L. acidophilus* has been shown to be able to survive and colonize the gastrointestinal tract (Kullen and Klaenhammer, 1999). Therefore, *Lactobacillus* species may have a beneficial effect past the rumen if they can withstand the rumen environment and pass into the intestines.

Propionibacterium freudenreichii is a gram-positive obligate anaerobe. *Propionibacterium* are naturally found in the rumen and produce propionic and acetic acid, two of the three major VFAs used as energy by the cow (Oshio et al., 1987). Various strains of *Propionibacterium* are able to affect ruminal fermentation by increasing molar proportions of ruminal propionate (Kim et al., 2000; Stein et al., 2006). 3 moles of lactate are converted to 2 moles of propionate, 1 mole of acetate, 1 mole of CO_2, and 1 mole of H_2O (Piveteau, 1999). Propionate is the main compound which is produced by *Propionibacterium* via the reduction of pyruvate. This involved several reactions; first oxaloacetate is formed by transcarboxylation. Oxaloacetate is

then reduced to succinate which is converted to propionate by methylmalonyl-CoA intermediates. Methylmalonyl-CoA is regenerated and can react with a new molecule of pyruvate continuing the cycle (Piveteau, 1999).

Propionibacterium are able to utilize lactate to help stabilize the rumen environment as described earlier. In a study done in sheep by Mackie et al. (1978) they observed that *Propionibacterium* accounted for 40-50% of the lactate utilizers when the animals were switched to a high concentrate diet. This was true even though the population number of *Propionibacterium* were very low, indicating that supplementation with this organism may help increase lactate utilization and propionate production in the rumen when feeding a high concentrate diet. Therefore, feeding *Propionibacterium* may help prevent the risk of acidosis by utilizing lactate, decreasing the amount of lactic acid in the rumen.

Supplementing both of these organisms in a DFM together may have a symbiotic effect on the dairy cow. Since *Propionibacterium* is a lactate utilizing bacteria, feeding in combination with a lactic acid producing bacteria can help avoid the accumulation of ruminal lactate (Stein et al., 2006). Also, when more lactate is available for *Propionibacterium* to utilize, propionate production increases inhibiting methane production by reducing the amount of hydrogen available. This aids in improving energy efficiency in the rumen (Krehbiel et al., 2003). McCowen et al. (1978) and Cheng et al. (1979a,b) found that *Lactobacillus* and *Propionibacterium* bacteria attached to the rumen wall of cattle. This infers that when feeding these organisms as DFMs, they may colonize on the rumen epithelial wall. In cattle, it is estimated that 1 –

2% of the total bacterial population resides on the rumen epithelium (Russell et al. 2002).

Bovamine® is a commercially available DFM product of Chr. Hansen (Hørsholm, Denmark) and is a combination of these two bacteria, *Lactobacillus acidophilus* strain LA51, and *Propionibacterium freudenreichii* strain NP24. More specifically, the product fed is manufactured specifically for dairy cattle and is marketed as Bovamine Dairy®. Studies specifically looking at Bovamine® in dairy cow diets are limited, but this product has the potential to beneficially impact animal performance by improving energy efficiency within the rumen.

1.4 GI Microbial Structure of Cattle

The GIT in any species is populated with a diverse microbial community that has an impact on energy efficiency in the host. Being ruminants, dairy cows have an enlarged foregut, the reticulorumen, where different microbial species are able to digest feedstuffs by anaerobic fermentation before entering the absorptive regions of the GIT (Russell, 2002). The digestion of plant polymers is very important in herbivorous animals. Ruminants are dependent on the microbial degradation of their feed to turn the polysaccharides provided by the diet, that cannot be digested by the host, into end products that are used as energy (Uyeno et al., 2015).

In mammals, most of the GIT bacterial community consists of two main phyla, Bacteroidetes and Firmicutes (Abnous et al., 2009). In cattle, the main GIT bacterial groups have been identified at the Genus level for up to 90% of the total community

(Uyeno et al., 2010). That leaves the remaining microbial population to still be unknown. The rumen is colonized with prokaryotes, protozoa, fungi and methanogenic archaea, but the bacterial population is most commonly studied as they are the most diverse group and represent more than half of the biomass (Martin, 1994). The development of high-throughput sequencing (HTS) techniques allow researchers to better understand microbial populations. With shorter run times and reduced cost, HTS allows users to quantify and identify microbial communities. Specifically, 16S rRNA gene sequencing has been used to better understand ruminal bacterial diversity (Whitford et al., 1998; Tajima et al., 1999; Kocherginskaya et al., 2002).

Proteins, vitamins, and short chain organic fatty acids are provided for the host by the microorganisms that inhabit the rumen. However, environmental factors have the ability to strongly influence and alter the composition and function of the rumen microbiota (Zoetendal et al., 2004). These factors could include stressors experienced by the animal such as heat stress and calving. As well as management influences like diet composition, feeding practices, and so on. For example, the transition period is a critical phase in a cow's life. The amount of physiological stress she is under entering lactation increases the incidence of metabolic disorders (Drackley, 1999). In addition, the diet changes from a high forage-based ration to a high concentrate-based ration which predisposes the cows to SARA (Fairfield et al., 2007). Feeding a DFM may be a strategy to stabilize the rumen response to these factors.

1.5 Effects of Supplementation with DFM on the Performance of Lactating Dairy Cows

Bovamine®

A study by Boyd et al. (2011) showed the inclusion of Bovamine® in dairy cow rations increased milk and protein yield but showed no differences in dry matter intake (DMI). Bovamine® supplementation also increased energy corrected milk (ECM) but did not affect energy efficiency. ECM is a value used to standardize production values to compare production across different cows, different dairies, and so on. Similarly, West and Bernard (2011) reported an increase in milk yield when supplementing Bovamine® without affecting DMI. Milk yield increasing while not affecting DMI suggests that rumen function was improved. O'Neil et al. (2014) reported that Bovamine® decreased DMI without affecting milk or ECM production, resulting in an improvement in milk production efficiency and ECM production efficiency. Ferraretto and Shaver (2015) observed a trend for DMI to be lower in cows receiving the Bovamine® treatment and reported no differences in milk yield, milk fat and protein. Collectively, these studies suggest that Bovamine® improves productive efficiency, either through increasing milk yield without increasing DMI or through decreasing DMI while maintaining milk yield.

In addition, the effect of these bacteria on digestibility and ruminal VFAs have been studied. Boyd et al. (2011) observed that supplementing Bovamine® improved

apparent digestibility of CP, NDF and ADF. Ferraretto and Shaver (2015) evaluated total tract starch digestibility and reported no difference in Bovamine® compared to control cows. Total ruminal VFAs in cows supplemented with Bovamine® increased in a study by Osman et al. (2012). The ability of these bacteria to improve digestibility and beneficially alter VFA balance in the rumen is critical when trying to improve feed efficiency. Overall, studies focused on feeding Bovamine® to dairy cows have varied results on performance measures.

Lactobacillus acidophilus LA747 & *Propionibacterium freudenreichii* PF24

In a study by Raeth-Knight et al. (2007), they used the same bacteria that comprises Bovamine® but different strains. They found no change in DMI or milk yield between control and DFM supplemented cows. In addition, supplementing DFM to mid-lactation dairy cows had no effect on apparent total tract digestibility of DM, NDF, CP, and starch. They did observe a trend for increased ruminal propionate concentration in DFM supplemented cows but did not observe a significant difference in total VFA concentration.

Propionibacterium

Weiss et al. (2008) conducted a study using a *Propionibacterium* strain and observed that cows with the DFM treatment had lower DMI, resulting in an increase in the efficiency of converting DM to milk yield. They suggest this is due to decreased acetate and increased propionate levels in the rumen improving fermentation. Francisco

et al. (2002) supplemented a *Propionibacterium* culture to early lactation cows and reported that DM consumption also decreased while milk yields were similar between the control and treatment groups. Stein et al. (2006) reported that when feeding *Propionibacterium* ruminal propionate concentrations increased. Being a lactate utilizing bacteria, *Propionibacterium* strains have the potential to improve the ruminal environment when included in dairy cow rations.

Other Organisms

Other microorganisms besides the bacteria that comprise Bovamine® have been studied as DFM supplements in dairy cow diets. For example, Sun et al. (2012) found no improvement in DMI but supplementing *Bacillus subtilis* improved milk composition and yield. Cows supplemented with *Bacillus subtilis* were able to utilize more nutrients on the same amount of feed which increased production efficiency. They also found including this DFM promoted the growth of total ruminal bacteria. Peng et al. (2012) reported an increase in ruminal propionate concentrations when feeding a *Bacillus subtilis* DFM. These results indicate that *Bacillus subtilis* has the potential to be used beneficially as a DFM in dairy cow diets.

Nocek et al. (2003) fed transition cows a DFM consisting of two *Enterococcus faecium* strains. Postpartum, the DFM supplemented cows had increased DMI, milk yield, and milk protein content compared to control cows. Another study by Nocek and Kautz (2006) found similar results where cows supplemented with DFM had increased DMI and milk yield but found no difference in milk protein percent or yield. AlZahal et

al. (2014) supplemented a DFM consisting of both *Enterococcus faecium* and *Saccharomyces cerevisiae* and reported no differences in DMI, milk yield, milk fat and protein between treatment and control cows. AlZahal et al. (2014) also observed that cows supplemented with a DFM had lower fecal starch content but showed no difference in NDF digestibility.

1.6 Summary

In dairy operations the largest cost of producing milk is the cost of feed. If dairy farmers are able to raise more efficient cows, that produce more milk per unit of feed, they can improve their profitability. Supplementing a DFM into the dairy cow diet can assist in improving efficiency. Bovamine® is a DFM that consists of two bacteria, *Lactobacillus acidophilus* and *Propionibacterium freudenreichii. P. freudenreichi* is able to utilize lactate produced by *L. acidophilus* to produce propionate, a precursor for glucose synthesis. Increasing propionate production should increase the energy available for the cow. If the dairy cow has more energy to put towards production, efficiency should increase.

The results of including a DFM in dairy cow diets to improve lactation performance or efficiency are still varied throughout the literature. There is still much debate on dosage, feeding time and frequency, and the strains used since there are many factors that influence how an animal will respond to DFM supplementation. Conflicting findings suggest that further research is necessary to develop a better understanding of how DFMs work and which strains elicit a response *in vivo*.

Chapter 2

OBJECTIVES

The DFM Bovamine® produced by Chr. Hansen is designed to improve digestive efficiency in dairy cattle. In Experiment 1, cows were fed their respective treatments, BOV or CON, for a 10-week period. Cows on the BOV treatment were supplemented with Bovamine® as a twice daily topdress. Bovamine® (1.5×10^9 CFU/feeding, 3×10^9 CFU/head/d, 28 g/head/d) was mixed with a ground corn grain carrier (100 g/feeding, 200 g/d). Cows on the CON treatment received 100 g corn grain at each time as well as 14 g of a 50/50 mixture of dried distiller's grains and calcium carbonate. Samples were taken throughout the experiment to observe both short and long-term benefits of supplementing Bovamine® in dairy cow rations. In this experiment we evaluated the effect of Bovamine® on milk production, total tract nutrient digestibility, and feed efficiency of early lactation dairy cows.

Experiment 2 evaluated the potential for Bovamine® to stabilize the rumen environment during an abrupt diet change to a high starch ration. This was done to observe if feeding Bovamine® could compensate for any undesirable effects due to errors in feeding that may occur on a dairy farm. This experiment was carried out as a crossover design with two 6-week periods, where cows were assigned the BOV or CON treatment for the first period and switched to the opposite treatment for the second period. Throughout the first 5 weeks of each period cows were fed a standard lactating cow ration that contained 23.8% starch. During week 6 of each period all cows were

challenged with an abrupt ration shift that contained 31.1% starch. Rumen and fecal samples were taken to observe any reduced changes in rumen pH, rumen VFA, rumen in situ digestibility, microbial composition, and fecal starch of cows on the Bovamine® treatment compared to control cows.

Chapter 3

EXPERIMENT 1: EFFECT OF BOVAMINE® ON PERFORMANCE AND NUTRIENT DIGESTIBILITY IN HIGH PRODUCING, EARLY LACTATION HOLSTEIN COWS.

3.1 Introduction

When fed to dairy cattle DFMs may help to improve feed efficiency. This would allow producers to supplement a DFM in their rations to reduce the amount of feed per unit of milk produced, improving the efficiency of the animals. This is economically beneficial to dairy farmers in order to increase production income relative to feed costs. Frequently, bacterial DFMs consist of a lactic acid producing bacteria fed in combination with a lactic acid utilizing bacteria, two of these commonly being *Lactobacillus* and *Propionibacterium* species. For example, *Lactobacillus acidophilus* produces lactic acid and *Propionibacterium freudenreichii* utilizes the lactic acid producing propionate. Since propionate is a major VFA that the cow uses for energy, increasing the production of propionate should increase the amount of energy available to the animal which she can then put towards production (Kim et al., 2000). Previous work on feeding these two bacteria has had variable results on productive efficiency. Feeding these DFMs have increased production (Nocek et al., 2003; Boyd et al., 2011; West and Bernard, 2011; Sun et al., 2012), decreased DMI (Francisco et al., 2002; Weiss et al., 2008; O'Neil et al., 2014), a combination of both (Nocek and Kautz, 2006), or neither (Raeth-Knight et al., 2007; AlZahal et al., 2014). These changes have resulted in an increase in productive efficiency in some studies (West and Bernard, 2011; O'Neil et al., 2014) but not others (Nocek and Kautz., 2006; Raeth-Knight et al., 2007; Boyd et

al., 2011; Osman et al., 2012, Ferraretto and Shaver, 2015). While some have shown increased performance or productive efficiency when feeding DFMs, results are still inconsistent.

It is suggested that DFMs are able to modify rumen microbial populations and alter rumen fermentation profiles to improve diet digestibility (Yoon and Stern, 1995; Krehbiel et al., 2003). But the effect of *L. acidophilus* and *P. freudenreichii* on nutrient digestibility has not been extensively studied. A study by Boyd et al. (2011) showed that supplementing these bacteria to dairy cows improved apparent digestibility of CP, NDF and ADF, but did not affect DM digestibility. In contrast, Ferraretto and Shaver (2015) and Raeth-Knight et al. (2007) reported no difference on apparent DM, NDF, CP, and starch digestibility. Therefore, it is difficult to determine if DFMs help improve nutrient digestibility since findings have been inconsistent and the mechanisms are still unknown.

Bovamine® is a commercially available DFM produced by Chr. Hansen that consists of two bacteria that symbiotically work with each other, a lactic acid producing bacteria *Lactobacillus acidophilus* strain LA51, and a lactate utilizing bacteria *Propionibacterium freudenreichii* strain NP24. In this first experiment we hypothesized that supplementing Bovamine® to early lactation dairy cows would increase feed efficiency as would be evidenced by increased milk yield, reduced intake with maintained milk yield, and/or increased nutrient digestibility as compared to unsupplemented cows.

3.2 Materials & Methods

Animals, treatments, and rations.

Thirty early lactation multiparous Holstein dairy cows were housed in a 30-cow deep sand bedded freestall barn and fed using individual Calan gates. At the start of the trial, mean (± SD) days in milk were 75 (± 32) and milk yield was 49 (± 6) kg/d. Cows were fed once daily (~0800 h) for ad-libitum intake and refusals were removed and weighed daily for measurement of daily intake. Cows were milked twice daily (~0430 and 1600 h) with milk weights recorded at each milking. Cows were weighed monthly on two consecutive days.

The experiment was conducted over 12 weeks, with a 2-week baseline period followed by a 10-week experimental period. During the baseline period, all cows were fed a total mixed ration without DFM (Table 1). At the end of the baseline period, cows were blocked by production and days in milk and randomly assigned to one of two treatments according to a randomized block design. During the treatment period, cows on CON treatment continued to be fed the ration without DFM while cows on the BOV treatment received the same ration but supplemented with Bovamine® (3×10^9 CFU/head/d). Cows remained on their respective ration until the completion of the 10-week experimental period.

Cows on the BOV treatment were supplemented with Bovamine® as a twice daily topdress given ~8 am and 5 pm. Bovamine® (1.5×10^9 CFU/feeding, 3×10^9 CFU/head/d, 28 g/head/d) was mixed with a ground corn grain carrier (100 g/feeding, 200 g/d). Cows on the CON treatment received 100 g corn grain at each time as well as

14 g of a 50/50 mixture of dried distiller's grains and calcium carbonate. Topdress treatments were prepared once weekly in the lab by weighing the corn grain and Bovamine® or corn grain and distillers grains/calcium carbonate into individual bags labeled with each cow number (14 bags per cow per week, with one bag for each cow for each am and pm feeding).

Milk sampling, feed sampling, and efficiency calculations.

Milk samples (720 total) were collected at both milkings on a consistent day each week and were analyzed for lactose, protein, fat, SCC and MUN by NIR using a Milkoscan System 4000 (Dairy One, Ithaca, NY). Energy corrected milk (ECM) was calculated as (0.327 * milk lbs.) + (12.97 * fat lbs.) + (7.21 * protein lbs). 3.5% fat corrected milk (3.5FCM) was calculated as (0.432 * milk lbs.) + (16.216 * fat lbs.). Feed efficiency was calculated as milk/DMI, ECM/DMI, and 3.5*FCM/DMI.

Samples of wet forages were collected three times a week and dry feeds collected once weekly. Subsamples were used for dry matter determination (55°C for 48 h) and the remainder was frozen until composited at 2-week intervals. Dried composite samples (50 total) were mailed to Cumberland Valley Analytical Services (CVAS) for nutrient analysis using the standard analysis package (DM, CP, ADF, NDF, ash, NFC, TDN, NEL, and minerals) plus starch (Table 2).

Digestibility and fecal starch measures.

A subset of 14 multiparous cows (7 per treatment) were used for measurement of total tract apparent nutrient digestibility at the end of the baseline period (wk -1), at 1 and 2 weeks following the start of the treatment period (wk 1, 2), and every 2 weeks thereafter (wk 4, 6, 8, 10). On the last day of each of those weeks, fecal grab samples were collected at 0900, 1500, 2100, and 0300 h and frozen until composited into 1 sample per cow per day and re-frozen. Two independent TMR samples were also collected from the morning feeding on the day of fecal sampling by using a small shovel to collect TMR from 10 different feed bins into a 5-gallon bucket. Independent samples were sequentially mixed and halved and frozen until analysis. Composite fecal samples (98 total, 1 for each of the 14 cows for each of the 7 days of digestibility measurements) and individual TMR samples (14 total, independent duplicates from each of the 7 days of digestibility measurements) were mailed to CVAS for measurement of CP, NDF, ADF, OM, starch, and 240 hour undigested NDF (uNDF). Total tract apparent digestibility of CP, NDF, ADF, starch, and OM were determined for each of the 14 cows by using uNDF as an internal marker.

For the remaining 16 cows, fecal samples were collected on the same dates and times and frozen until composited into 1 sample per cow per day and re-frozen. Composite fecal samples were mailed to CVAS for measurement of fecal starch.

At the time of fecal collection on each day fecal score (1=liquid to 5=extremely well formed) was evaluated on all 30 cows. This allowed for us to use the entire set of 30 cows to evaluate the effect of Bovamine® on fecal starch and fecal score.

Statistical analysis.

Weekly means of dry matter intake and milk yield were calculated for each cow. Milk composition data for each day of sampling was calculated as the mean of the am and pm sampling results.

One cow (cow 51) was removed from the experiment during week 8 due to clinical mastitis. Her data through week 7 was included in all statistical analyses except when indicated otherwise. One cow (BOV) had low milk fat and protein yields throughout the experiment, low intakes during the beginning of the experiment, and was sometimes identified as an outlier by the univariate procedure of SAS. An additional five cows (3 CONl, 2 BOV) had chronically high SCC. Statistical analyses of performance data were completed both including and excluding the data of those six cows.

Weekly measures of intake, milk yield, and milk composition were analyzed using the GLIMMIX procedure of SAS. Treatment, week, and the interaction of treatment by week were included as fixed effects. Data collected during the last week of the baseline period was included as a covariate. The "random _residual_" statement was used to indicate repeated measures, the subject was cow nested within treatment, and an autoregressive covariance structure was used. Cow was included as a random effect. Differences were determined by using the "pdiff" option of the "lsmeans" statement.

Fecal score, fecal starch, and apparent nutrient digestibility data were evaluated using the same model except that there were fewer weeks included in the model (fecal score was determined during the baseline period and during weeks 1, 2, 4, 6, 8, and 10).

Body weights collected at the end of the baseline period and at the end of weeks 5 and 10 were analyzed using the GLIMMIX procedure of SAS. Treatment, week, and the interaction of treatment by week were included as fixed effects. Data collected during the last week of the baseline period was included as a covariate. Cow was included as a random effect.

3.3 Results & Discussion

Performance

Weekly means of intake, milk yield, milk composition, and body weight were evaluated for all cows over the course of the experiment (Table 5). Treatment or the interaction of treatment by week did not affect any of the measures evaluated. There was a trend for a treatment by week interaction on DMI ($P = 0.09$). This was due to greater intakes by CON as compared to BOV during week 4 (27.3 vs 25.3 kg/d, $P = 0.05$) and week 5 (25.9 vs. 28.4 kg/d, $P = 0.01$), data not shown. Presented in Table 6 are the results excluding cows that were identified as outliers or high SCC cows (3 CON and 3 BOV). Treatment alone did not affect any measures, but the interaction of treatment and week affected DMI ($P = 0.01$) and fat percent ($P = 0.03$) and tended to affect milk/DMI ($P = 0.06$). For DMI, this was driven by greater intakes for CON than BOV during week 5 ($P = 0.004$) and a tendency during week 4 ($P = 0.07$; Figure 7). For milk fat percent, the interaction was driven by BOV having numerically greater milk fat percentages during weeks 2 and 3 than CON and CON having numerically greater milk fat percentages during weeks 4 to 10, with this difference approaching significance at

week 7 ($P = 0.07$; Figure 8). The tendency for the interaction of treatment by time on milk/DMI was due to greater efficiency for BOV than CON during week 5 (1.78 kg/kg vs. 1.65 kg/kg, $P = 0.04$; data not shown). No differences were observed between treatments for ECM/DMI or FCM/DMI.

In this experiment, supplementing high producing early lactation dairy cows with Bovamine® did not affect cow performance (Table 5). It is suggested that supplementing cows with lactate producing and utilizing bacteria will provide a more constant production of VFAs which the cow can utilize for production (Nocek et al., 2003). Boyd et al. (2011) showed the inclusion of Bovamine® in dairy cow rations increased milk yield. West and Bernard (2011) also reported an increase in milk yield when supplementing Bovamine®. In contrast and in agreement with our experiment, others also have not found an increase in milk production when cows were fed Bovamine® (O'Neil et al., 2014; Ferraretto and Shaver, 2015).

In this experiment we expected BOV supplemented cows would have increased efficiency, either by an increase in milk yield or by decreasing DMI while maintaining milk yield. Measuring milk/DMI is one way to measure feed efficiency and is interpreted as pounds of milk produced per pound of dry matter consumed. West and Bernard (2011) found that Bovamine® supplemented cows were more efficient. In their study ECM tended to be greater in the DFM treated cows and the DFM group had numerically decreased DMI, resulting in Bovamine® treated cows having a greater ECM/DMI. O'Neil et al. (2014) also reported decreased DMI in Bovamine® treated

cows, resulting in an improvement in milk/DMI. This is opposed to Raeth-Knight et al. (2007) and Ferraretto and Shaver (2015) who reported no differences between Bovamine® and control treatments for energy efficiency, similar to this experiment.

Previous research has found that Bovamine® has not affected milk fat or protein percentages (Raeth-Knight et al., 2007; Boyd et al., 2011; West and Bernard 2011; Ferraretto and Shaver, 2015). However, Boyd et al. (2011) reported increased milk protein yield in Bovamine® supplemented cows. These findings were mainly due to an overall increase in milk yield, not protein percentage. Based on this work we hypothesized that the inclusion of Bovamine® would not have an impact on milk components, which it did not. Others have found effects on milk composition when feeding different DFMs. Nocek et al. (2003) reported an increase in milk protein yield in cows supplemented with yeast and *Enterococcus* strains. Work by McGilliard and Stallings (1998) who supplemented a DFM mixture of *Aspergillus oryzae, Bacillus subtillis, Lactobacillus acidophilus,* and a yeast culture found a decrease in milk fat percentage. Nocek and Kautz (2006) also demonstrated milk fat percentage decreased in cows supplemented with a yeast and *Enterococcus faecium* strains. Despite the difference in milk fat percentage, both of those studies observed similar milk fat yields between treatment and control groups. Though there are some exceptions, throughout the literature DFM supplementation does not typically change milk composition in lactating dairy cows

Fecal measures and nutrient digestibility

Results for fecal score, fecal starch, and apparent total tract nutrient digestibility are presented in Table 7. Fecal score and digestibility of DM, OM, CP, NDF, and ADF were not affected by treatment or the interaction of treatment by time. There was an interaction of treatment by week for fecal starch ($P = 0.01$). This was due to greater fecal starch for CON than BOV at week 2 (Figure 9). Apparent total tract starch digestibility tended to be increased for the BOV treatment compared to CON ($P = 0.051$).

Dairy producers and nutritionists often evaluate fecal score when making feed changes. Manure evaluation can give a visual interpretation of digestion of consumed feed. Fecal matter is scored on a scale of 1 to 5, 1 being liquid and 5 being extremely well formed. Using this scale, manure with a score of 3 is optimal. An excess of protein or starch leading to poor rumen fermentation and increased hindgut fermentation, as well as a lack of fiber can cause manure with a fecal score below 3. Higher fiber rations, or low-quality forages with poor digestibility, tend to cause a fecal score above 3 (Hall, 2002). In high producing lactating cow diets, manure scores may sometimes fall below the optimum because of the higher amount of concentrate that is fed. In this study we expected nutrient digestibility to be best for BOV cows. Therefore, we expected fecal scores for BOV cows to be closer to 3 than CON because reduced digestibility for CON would result in a higher or lower score. There was not a significant difference in fecal score between CON and BOV. CON and BOV cows had fecal scores around the optimal score of 3 (2.96 and 3.07 respectively), suggesting nutrient digestibility between the two groups were similar.

Due to the expense of nutrient digestibility analysis, analyses were only done on 14 cows in this experiment (7 per treatment). It was hypothesized that nutrient digestibility would be improved in BOV cows. It is suggested that supplementing DFMs in cattle improve rumen fermentation in turn improving digestibility (Krehbiel et al., 2003). In our study treatment did not affect digestibility of DM, OM, CP, NDF, and ADF. Although there was a tendency for BOV to have improved starch digestibility, it was biologically not very different (98.46% CON and 98.74% BOV). Raeth-Knight et al. (2007) also did not observe a significant effect of *Lactobacillus* and *Propionibacterium* supplementation on apparent nutrient digestibility of DM, OM, CP, NDF, ADF and starch. This is opposed to Boyd et al. (2011) who observed increased CP, NDF and ADF digestibility in Bovamine® cows as compared to control. In regard to other DFM supplementation in lactating dairy cows, Nocek and Kautz (2006) reported increased digestion of forage DM in cows supplemented with yeast and *Enterococcus* strain DFM. A study by AlZahal et al. (2014) showed improved starch digestibility in cows supplemented with an *Enterococcus faecium* and *Saccharomyces cerevisiae* DFM but did not observe any difference in apparent total tract nutrient digestibility of NDF.

Starch digestibility was only measured in a subset of animals in this experiment, but fecal starch content was evaluated in all 30 cows. Fecal starch can be used as an indicator of total-tract starch digestibility (Fredin et al., 2014). An increase in total-tract starch digestibility can increase milk yield, milk protein yield, and feed efficiency (Firkins et al., 2001). In our study fecal starch percent was not affected by treatment,

which supports the findings of the subset of cows where Bovamine® had a tendency to only modestly improve starch digestibility.

Conclusions

Overall, this experiment showed no benefits of feeding Bovamine® on milk production, feed efficiency, fecal score or fecal starch in high producing early lactation dairy cows. There was a tendency for BOV cows to have improved starch digestibility, but the magnitude of the difference was small (0.28%) and digestibility of other nutrients were not affected by treatment. Although some studies have shown that supplementing DFM improves milk production, component yield, or feed efficiency, results have been inconsistent. Differences among studies may be due to differences in inclusion levels, feeding protocols, and diet composition that can impact animal response to the DFM.

Chapter 4

EXPERIMENT 2: EFFECT OF BOVAMINE® ON RUMEN PH AND VFA, IN SITU DIGESTIBILITY, FECAL PH AND STARCH, AND RUMEN AND FECAL MICROBIOMES BEFORE AND FOLLOWING A HIGH STARCH FEEDING CHALLENGE.

4.1 Introduction

In the field, producers aim to feed their herd a consistent ration day after day. Dairy cows perform more efficiently when they are faced with minimal changes in their diet. Realistically this may not always be the case; feeding inconsistencies may arise for many reasons. For example, person to person variation if different individuals are making feed, forage changes, too much of a feedstuff being added, equipment malfunctions, weather and storage conditions can all result in mixing errors. A producer may choose to include a DFM in the ration to improve digestive function and rumen stability in the herd. One way DFMs can maintain rumen stability is by producing a steady level of lactate, which would allow lactic acid utilizing microbes to sustain a metabolically active population. This would allow these lactate utilizers to sequester more lactate when concentrations fluctuate due to diurnal feeding (Nocek and Kautz, 2006). Theoretically, if dairy cows are faced with a diet change, but are receiving a supplemented DFM in their ration, they should have less fluctuation in rumen and hindgut digestibility. This is especially important when mixing errors increase dietary concentrate. During this time, lactate production increases faster than the lactate utilization rate which can drastically drop rumen pH. Supplementing a DFM may help prevent ruminal acidosis (Nocek et al., 2000).

Bovamine® is a DFM that consists of a lactate producing bacteria as well as a lactate utilizing bacteria. It is predicted that these organisms work together to provide a steady dose of lactate in the rumen, continually stimulating the lactate utilizing bacteria to produce major byproducts like VFAs. Therefore, ruminal lactate should be rapidly utilized in the rumen, maintaining a steady pH. In this experiment we hypothesized that when faced with a high starch challenge, BOV cows would have a more stable rumen environment. This would result in less fluctuation in rumen pH, rumen VFA, in situ digestibility, fecal starch, and rumen and fecal microbiome compared to CON.

4.2 Materials & Methods

Animals, treatments, and rations.

Experiment 2 used 6 lactating Holstein dairy cows housed in tie stalls. Cows were fitted with rumen cannulas prior to the start of the experiment. The cows initially were comprised of 3 multiparous and 3 primiparous cows with mean (± SD) milk yield of 40 (± 8) kg/d and days in milk of 73 (± 32). One primiparous cow had to be removed from the experiment during week 2 of period 1. Due to lack of availability of replacement cannulated cows, she was replaced with a multiparous cow much later in lactation (377 DIM) and lower in milk (11 kg/d). The final set of 4 multiparous cows and 2 primiparous cows had a mean milk yield of 36 (± 15) kg/d and days in milk of 123 (± 129).

Cows were fed twice daily (~0900 and 1630 h) for ad-libitum intake and refusals were removed and weighed daily for measurement of daily intake. Cows were milked twice daily (~0430 and 1600 h) with milk weights recorded at each milking.

The experiment was conducted as a crossover design with two 6-week periods. During the first period, 3 cows were assigned to the CON and 3 cows were assigned to BOV. Cows on the BOV treatment were supplemented with Bovamine® as a twice daily topdress given ~9 am and 4:30 pm. Bovamine® (1.5×10^9 CFU/feeding, 3×10^9 CFU/head/d, 28 g/head/d) was mixed with a ground corn grain carrier (100 g/feeding, 200 g/d). Cows on the CON treatment received 100 g corn grain at each time as well as 14 g of a 50/50 mixture of dried distillers grains and calcium carbonate. Topdress treatments were prepared once weekly in the lab by weighing the corn grain and Bovamine® or corn grain and distillers grains/calcium carbonate into individual bags labeled with each cow number (14 bags per cow per week, with one bag for each cow for each am and pm feeding). According to the crossover design, cows were switched to the opposite treatment for the period 2.

Cows were fed one of two rations during the course of the experiment (Table 3). During the first 5 weeks of each period were fed a typical lactation ration balanced to contain 30% NDF and 25% starch (Table 4). During week 6, cows were abruptly switched to the same ration mixed with an additional 12.8 kg corn grain per 100 kg ration dry matter. The week 6 ration was formulated to contain 27.9% NDF and 30.5% starch and was used to simulate a mixing error that might occur on farm.

Milk and feed sampling.

During each period, milk samples (192 total) were collected at both milkings on day 7 of each week during weeks 1-5 and on days 1, 3, and 7 of week 6. Samples were analyzed for lactose, protein, fat, SCC and MUN by NIR using a Milkoscan System 4000 (Dairy One, Ithaca, NY). Samples of wet forages were collected three times a week and dry feeds collected once weekly. Feed sample composites (50 total) were generated for weeks 1-2, weeks 3-4, week 5, and week 6 of each period. Composites were analyzed using wet chemistry methods by Cumberland Valley Analytical Services (CVAS; Hagerstown, MD) for CP, NDF, ADF, starch, ash, and mineral content, and analyzed composition is presented in Table 2.

Rumen and fecal measures.

Rumen pH was continuously measured during weeks 5 and 6 in all cows using indwelling pH meters (DASCOR, Inc.). In addition, in situ rumen digestibility, rumen VFA, fecal pH, fecal starch, and rumen and fecal microbiome were determined during weeks 5 and 6.

In situ digestibility was measured at the end of week 5, beginning of week 6, and end of week 6. Dried and ground TMR was placed in Dacron bags (4.0 ± 0.1 g TMR in 10 × 20 cm bags) in the rumen and incubated in triplicate in each cow to evaluate dry matter disappearance after 6, 12, 18, and 24 h in the rumen. Timing of bag placement occurred such that bags were placed in the rumen at different times (4, 10, 16, and 22 h relative to feeding), but all bags were removed from the rumen at the same

time. A separate set of triplicate bags was subjected to rinsing but not ruminal incubation to correct for losses due rinsing alone.

At the time of bag placement (4, 10, 16, and 22 h relative to the am feeding), rumen and fecal samples were collected for measurement of rumen VFA (144 total samples), fecal pH (144 total measurements), and fecal starch (composited by cow and sampling day, 36 total samples). For measurement of fecal pH, 20 g of feces was added to 20 mL water in a 50 mL conical tube. The mixture was shaken vigorously for 20 seconds and the liquid squeezed through 4 layers of cheesecloth. The pH of the liquid was then measured using a portable pH meter (P771, Anaheim Scientific, Yorba Linda, CA). Samples collected for rumen VFA were stored at -20°C until VFA analysis using high-performance liquid chromatography. Fecal samples for starch determination were stored at -20°C until composited by cow within each sampling day. Starch content of fecal composite samples was analyzed at CVAS.

At the 10 h time point following the first feeding at the end of week 5 (w5d7), and the beginning (w6d1) and end of week 6 (w6d7) during periods 1 and 2, sterile samples of feces and rumen fluid were collected for microbiome analysis from each cow (72 total samples). Rumen fluid was collected from four different areas in the ventral rumen sac (~ 50 mL per site) and put into a sterile whirl-pak bag. Using a sterile funnel, rumen fluid was strained through two layers of sterile cheesecloth into a sterile bottle. 5 mL cryovials were filled approximately halfway with the filtered rumen fluid sample and were then stored at -80°C. This method was repeated for each cow. Feces was collected by palpating the rectum to obtain the fecal sample into a sterile beaker.

Using a sterile spatula, 5 mL cryovials were filled approximately halfway with the fecal sample and stored at -80°C. This method was repeated for each cow. Frozen microbiome samples were sent to RTLGenomics (Lubbock, TX) for sequencing

Microbiome analysis.

DNA extraction, library preparation, amplification and sequencing was done by RTLGenomics. The primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) were used to amplify the V4 and V5 hypervariable regions of the 16S rRNA. Samples were amplified for sequencing using the Illumina two-step process. The Illumina MiSeq (San Diego, CA) platform with the 2 × 250 base pair paired-end method was used for sequencing.

Sequence processing was done by Mark Miller at Phaseolus Consulting (Wyndmoor, PA). FASTQ files generated by RTLGenomics were imported into a Qiime 2 (v2018-2) paired-end "sequences with quality" artifact. The number of reads for each sample are illustrated below in Figure 1. The applications FASTQC and MultiQC were used to build a per-nucleotide/per-sample quality histogram of the raw data (Figure 2). The quality scores of these FASTQ files were consistently good. Phred quality scores indicate the probability that a given base is called incorrectly by the sequencer. For this data, almost all of the per-nucleotide Phred quality scores were over 30, indicating a base-call accuracy rate of 99.9% or greater (Ewing and Green, 1998).

Sequence artifacts were run through the Divisive Amplicon Denoising Algorithm 2 (DADA2), which is a model-based approach for correcting amplification

errors without constructing operational taxonomic units (OTU) (Rosen et al., 2012). After sequences were denoised and tabulated, three FASTQ files were chosen to select for the appropriate DADA2 settings. Trimming and truncation values of 20 and 240 nucleotides were selected in agreement with Figure 2. DADA2 was run on a 32-core, 60 GB AWS "c3.8xlarge" cloud server. DADA2's thread count parameter was set to 32 and the number of reads used for learning error patters was increased from 1^6 to 2^6. For the other parameters the default settings were used.

After removing the low quality and noisy reads, DADA2 tabulates the remaining reads in each sample as OTUs at 100% identity. An OTU is a marker sequence that is representative of the observed sequences from one or more highly related individual(s) (Blaxter et al., 2005). After DADA2 tabulation, OTUs were classified taxonomically. Figure 3 plots the total number of OTU features per sample against the number of reads per sample. Most of the samples retained ~50% of the reads after the DADA2 steps. DADA2 detected sequences from a total of 8,100 different OTUs.

Next, two filters were applied to the samples. The first filter discarded any sample whose total OTU count was less than two standard deviations below the mean of all samples. That minimum for this data was 4,162; therefore two samples were deleted: Cow225-Pd2-w5d7-Fluid and Cow148-Pd2-w6d7-Fluid (rumen fluid samples from cow 225 in period 2, week 5, day 7 and cow 148 in period 2, week 6 day 7). The second filter that was applied required a total count of at least 3 OTUs across all samples (either in a single sample or across multiple samples). Any OTUs that appeared only once or twice total were discarded as they may have represented sequencing errors. This

filtering removed 986 of 1,111,911 tabulated reads in the 8,100 different OTUs that were identified. Figure 4 depicts counts of OTUs, and those left of the dotted line (n=433) were removed, leaving a total of 7,667 remaining OTUs.

The filtered sequences were then input into Qiime 2's naïve Bayes classifier, to identify what organisms were present in the samples. The reference file used in the classifier was constructed from GreenGenes version 18.8 clustered at 99%, the highest similarity level provided. The naïve Bayes classifier was run with a confidence cutoff of 0.9 (Figure 5). The majority of these classifications had a high confidence with a sharp peak at 99 – 100%.

The output of the DADA2 tabulation and the taxonomic classification of OTUs were merged together with a sample metadata file to obtain a richly annotated .biom file. Further analyses were performed by importing the .biom file and the phylogenic tree into a script written in R language using Bioconductor's Phyloseq library. The R script added an additional OTU feature filtering step to remove those that had been classified as rRNA with mitochondrial or chloroplast origin (total of 131 features).

Almost all of the OTUs can be classified to Order, 75% could be classified to Family, 42% could be classified to Genus, and 5% could be classified at the Species level (Figure 6). Due to lower specificity at the Genus and Species classifications, results are discussed at the order and family level. All of the OTUs from this data set were aggregated into 82 families.

Animal number justification.

The number of animals was limited to 6 due to the expense associated with rumen cannulation, rumen pH boluses and microbiome sequencing. Because this experiment was designed as a crossover, the effective number of animals per treatment is 6, as each cow received each treatment. Based on typical standard errors we observe, we expected 6 animals to be sufficient to detect differences of 10 mM, 0.2, and 0.25 in rumen VFA, rumen pH, and fecal pH, respectively.

Statistical analysis.

Weekly means of dry matter intake and milk yield were calculated for each cow. Milk composition data for each day of sampling was calculated as the mean of the am and pm sampling results.

Weekly measures of intake, milk yield, and milk composition were analyzed using the GLIMMIX procedure of SAS. Treatment, week, treatment sequence, period, parity, and the interactions of treatment by week and treatment by parity were included as fixed effects. The "random _residual_" statement was used to indicate repeated measures, the subject was the interaction of treatment and cow, and an autoregressive covariance structure was used. Cow within sequence was included as a random effect. Differences were determined by using the "pdiff" option of the "lsmeans" statement. Due to the low production of the one cow that was added to the study as a replacement (cow 138), her data were excluded when evaluating performance.

Daily mean intake, milk yield, and milk composition from day 7 of week 5 and days 1, 3, and 7 of week 6 were separately analyzed to determine any short-term effects

of high starch feeding. Those data were analyzed using the GLIMMIX procedure of SAS as described for the weekly means, except that the week term was replaced by an indicator of day within week.

Fecal starch was also analyzed using this model except that the days included were only day 7 of week 5, day 1 of week 6, and day 7 of week 6.

In situ disappearance, fecal pH, and rumen VFA were analyzed by the GLIMMIX procedure of SAS. Treatment, day, hour, treatment sequence, period, parity, and the interactions of treatment by hour, treatment by day, treatment by day by hour, and treatment by parity were included as fixed effects. The "random _residual_" statement was used to indicate repeated measures, the subject was the interaction of treatment and cow, and an autoregressive covariance structure was used. Cow within sequence was included as a random effect. Differences were determined by using the "pdiff" option of the "lsmeans" statement.

Rumen pH data were used to calculate daily values for mean pH, minimum pH, maximum pH, minutes/d below pH 5.8, and area/d below pH 5.8. Those results were evaluated using GLIMMIX and a model that included the fixed effects of treatment, day, week, parity, period, and sequence, and the interactions of week by treatment, day by treatment, day by week, and day by week by treatment.

Beta diversity analysis was performed on the microbiome samples using the phyloseq package in R to allow pairwise comparisons between samples. The DESEQ2 procedure was used to independently evaluate fecal and rumen samples using a general linear

model. Effects of treatment (CON or BOV) and ration starch content (low during week 5 day 7, and high during week 6 day 1 and week 6 day 7) were included in the model.

4.3 Results & Discussion

Performance

Weekly means of intake, milk yield, and milk composition for all cows (excluding low producing cow 138) over the course of the experiment were evaluated and results are presented in Table 8. Treatment did not affect any measures except that lactose percentage tended to be greater in BOV (4.86 vs. 4.81%, $P = 0.08$). The interaction of treatment by time did not affect any measures, but there was a trend for an interaction of treatment by parity for milk protein yield ($P = 0.06$). That interaction was due to similar milk protein yields for primiparous cows on both treatments, but numerically higher yields for multiparous cows fed BOV compared to CON (Figure 10).

Milk samples were collected on day 7 of week 5, just prior to the shift to the high starch diet, and on days 1, 3, and 7 of week 6 following the switch to the high starch ration. Milk components from each of those days as well as corresponding milk yields and intake were statistically evaluated to determine effects of Bovamine® during the shift to the higher starch ration. Results of that model while excluding cow 138 are presented in Table 9. Treatment or the interaction of treatment and day did not affect DMI or milk yield, but there was a tendency for an interaction of treatment and day on milk/DMI ($P = 0.06$). For milk/DMI, BOV cows were numerically more efficient than

CON cows on day 7 of week 5 and day 3 of week 6 whereas CON cows were numerically more efficient on day 1 of week 6 (Figure 11). Protein yield and percentage were not affected by either treatment or the interaction of treatment and day. There was a tendency for an interaction of treatment by day for fat % ($P = 0.10$) that was due to a tendency for greater milk fat percentage for BOV on day 1 of week 6 but no differences on the other days (Figure 11). Milk fat yield was affected by the interaction of treatment by day ($P = 0.02$) and treatment by parity ($P = 0.05$; Figure 11). These effects culminated in increased fat yield for BOV cows ($P = 0.002$), due to numerically greater milk fat yields for BOV on day 7 of week 5 and day 1 of week 6 and numerically greater milk yields of primiparous cows fed BOV.

As for experiment 1, we hypothesized that Bovamine® would increase feed efficiency as would be demonstrated by increased milk yield, maintained milk yield with decreased DMI, and/or increased milk/DMI. We expected these differences to be greatest following the abrupt shift to the high starch ration. As indicated above, Bovamine® may have helped to stabilize milk fat following the ration shift, but overall impacts were minimal. As described in Experiment 1, bacterial DFMs do have the potential to increase milk production and feed efficiency, though responses are highly variable across studies. Our primary objective in this study was to observe the impact Bovamine® had on ruminal fermentation characteristics when cows were abruptly switched to a high starch ration, and as a result our study was underpowered to detect performance responses.

Rumen pH, in situ digestibility, and VFA

Daily mean rumen pH variables were analyzed, and results presented in Table 10. Week affected minimum pH ($P = 0.05$) and tended to affect mean pH ($P = 0.07$). Interestingly, daily mean pH was 6.28 in week 5 and 6.35 in week 6. This is counter to our expectation that the higher starch ration fed during week 6 would decrease rumen pH. Minimum pH followed the same pattern and was 5.82 in week 5 and 5.90 in week 6. It is commonly known and reported that changing the proportion of forage and concentrate in the diet of cattle affects rumen pH, but this was not observed in our study. We were expecting higher rumen pH with the BOV treatment, particularly following the change to the high starch ration, but this was also not observed. Results from other studies with *Lactobacillus* species have shown decreased changes in pH in acidosis challenged cows, suggesting reduced risk of subacute ruminal acidosis (Huffman et al., 1992; Nocek et al., 2000). However other reports have not found an effect of DFM on rumen pH. When supplementing a *Propionibacterium* DFM, Kim et al. (2000) and Ghorbani et al. (2002) reported no effect on ruminal pH. Nocek et al. (2002) measured rumen pH when feeding different levels of a DFM treatment consisting of *E. faecium, L. plantarum,* and *S. cerevisiae.* At the lowest DFM inclusion rate (1 x 10^5 cfu/mL of rumen fluid), ruminal pH increased. Higher levels of DFM (1 x 10^6 cfu/mL of rumen fluid and 1 x 10^7 cfu/mL of rumen fluid) decreased ruminal pH. They stated that the production of acid increased faster than its utilization at the higher inclusion levels suggesting that there may be a threshold of acid production that many no longer stimulate the acid utilizing microbes.

Rumen fluid samples were collected at 4, 10, 16, and 22 hours after the morning feeding on day 7 week 5, day 1 of week 6, and day 7 of week 6 for measurement of rumen organic acids (Table 11). Treatment and the interaction of treatment by time did not affect ruminal VFAs. As expected, hour (4, 10, 16, and 22 after feeding) affected all measures ($P \leq 0.002$). Interestingly, day (week 5 day 7, week 6 days 1 and 7) affected all measures ($P \leq 0.01$) except for rumen lactate and the ratio of acetate to propionate, indicating that the higher starch feeding during week 6 did impact rumen variables. Across all times, LSmeans of total rumen VFA were 91 mM on day 7 of week 5, 109 mM on day 1 of week 6, and 119 mM on day 7 of week 6 (Figure 12a). Total VFA on day 7 of week 5 was lower than the other days ($P < 0.001$) and week 6 day 1 tended to be lower than week 6 day 7 ($P = 0.08$). Although we did not observe a change in rumen pH, the increase in ruminal VFAs indicate that the additional concentrate in the diet challenge increased rumen starch digestion.

Changing the diet has a cascading effect on rumen metabolism, resulting in changes in organic acid profiles (Wolin & Miller, 1997). In this study we hypothesized that BOV cows would have a more stable rumen environment when animals were switched to a high starch ration as would be evidenced by reduced changes in ruminal VFAs following the switch to the high starch diet. Based on our results, BOV did not improve ruminal stability because of the lack of treatment effects on ruminal organic acids or rumen pH. The lack of a significant effect of treatment on lactate and propionate concentrations indicate that supplemented bacterial strains may not have been able to remain sufficiently active in the rumen to alter ruminal fermentation. In

order to survive, the Bovamine® bacteria need to be in a favorable environment. *L. acidophilus* thrive at a pH of 5.50 – 6.0 (Altermann et al., 2005). The average pH observed in our study ranged from 5.8 - 6.8 with an average of 6.3. While this range is above the optimal conditions for *L. acidophilus*, they are still known to survive under these conditions but with less functionality (Altermann et al., 2005). In our study, no differences in ruminal propionate proportions were observed which may indicate why milk production was similar between the CON and BOV cows in Experiment 1. This is in agreement with Raeth-Knight et al. (2007) who also did not find a difference in total VFA concentration or milk yield between control and Bovamine® treatments. In opposition, Osman et al. (2012) found that feeding Bovamine® increased total VFA concentrations in the rumen but did not report changes in milk yield or efficiency in Bovamine® supplemented cows.

Other studies have measured the effect of different bacterial DFMs on the rumen organic acid profile. When feeding a *Propionibacterium* DFM, Kim et al. (2000) observed no effect on lactate or ruminal organic acids with supplementation. Ghorbani et al. (2002) also fed a *Propionibacterium* DFM and found no effect on ruminal concentrations of lactate, total VFA, propionate, acetate, valerate, isobutyrate, and isovalerate, or the ratio of actetate:propionate. But DFM supplemented cows did have greater concentrations of ruminal butyrate. However, Stein et al. (2006), Weiss et al. (2008) and Peng et al. (2012) observed a significant increase in ruminal propionate concentrations when feeding *Propionibacterium* or *B. subtilis* DFM.

When switched to a highly fermentable diet, VFA production increases in the rumen, and this likely occurred in our study as suggested by the increase in rumen VFA concentration (Figure 12a). Usually this increases total metabolizable energy for the animal which then increases production or productive efficiency (Penner et al., 2010). Despite the increase in rumen VFA, there was no effect of the high starch ration on production or productive efficiency, but again the period of high starch feeding was short, there were low animal numbers, and animals were in mid to late lactation. While some of the DFM studies mentioned above either increased or did not have an effect on ruminal organic acids, it is important to consider the different diet compositions and organisms that were used in each of these trials. Diet composition is the biggest factor that affects ruminal fermentation variables, and response to DFM is likely highly dependent on diet.

In situ digestibility following 6, 12, 18, and 24 hours of rumen incubation was also determined on the days rumen fluid samples were taken (Table 11). Treatment and the interactions of treatment by time did not affect in situ digestibility, but there was an effect of day ($P < 0.001$, Table 11) and hour (6, 12, 18, and 24 hours of incubation) ($P < 0.001$, Table 11). To visualize the effect of day, LSmeans for in situ digestibility measured following each of the four time points are presented in Figure 12b. The day effect for in situ digestibility was due to greater digestibility on day 7 of week 6 than digestibility measured on day 5 of week 7 or day 1 of week 6 ($P \leq 0.02$). To our knowledge, no work has been done to look at in situ digestibility in lactating dairy cows supplemented with Bovamine®. In Experiment 2 we hypothesized that BOV cows

would have increased in situ digestibility, particularly following the high starch challenge, as compared to CON. Nocek et al. (2002) measured the effect of DFM dosage on in situ digestibility of high moisture ear corn, haylage, and corn silage. In their study they used a combination of *Enterococcus, Lactobacillus and Saccharomyces* supplemented at 10^5, 10^6 or 10^7 cfu/mL rumen fluid. They found that cows supplemented with DFM at 10^5 CFU had higher digestion rates of high moisture ear corn and corn silage compared to the 10^6 and 10^7 doses, respectively. While the findings of this study are not in direct relevance to our experiment, it does show the potential of a DFM to modify in situ digestion kinetics. In our experiment, BOV supplemented cows did not have improved TMR in situ digestibility as compared to control cows. Ghorbani et al. (2002) also found that treatment with a *Propionibacterium* and *Enterococcus* DFM did not affect 24 hour in situ disappearance in beef steers. This is in agreement with our findings from Experiment 1, and with Raeth-Knight et al. (2007) where Bovamine® supplemented cows did not have improved apparent total tract nutrient digestibility of DM, OM, CP, NDF, ADF and starch. On the other hand, Boyd et al. (2011) reported increased digestibility of CP, NDF, and ADF in Bovamine® supplemented cows.

Fecal pH and starch

Fecal samples were collected at 4, 10, 16, and 22 hours after feeding on week 5 day 7, and week 6 days 1 and 7. Fecal pH was measured at all of those time points and remaining fecal samples were composited by day for each cow for measurement of

fecal starch. There were no effects of treatment on fecal pH (Table 12). There was an effect of day ($P = 0.002$), and fecal pH was lower on day 7 of week 6 (6.72) than on either of the other days (6.86 and 6.89 for week 5 day 7 and week 6 day 1, respectively). Fecal starch was affected by both treatment ($P = 0.02$) and day ($P = 0.01$). Fecal starch was greater for BOV than CON. The day effect was due to fecal starch being lower on week 5 day 7 (1.79%) than on week 6 day 7 (2.72%, $P = 0.002$), and neither of those days was different from day 1 of week 6 (2.26%). Though there was not an interaction of treatment by day ($P = 0.53$), the treatment difference appears to be largely driven by effects during week 6 (Figure 13). Together, these results suggest that the higher starch ration fed during week 6 reduced total tract starch digestibility, and that Bovamine® actually reduced starch digestibility, particularly following the transition to the high starch ration.

In this experiment, we hypothesized that BOV cows would have improved starch digestibility prior to and following the switch to the high starch ration. Counter to Experiment 1, fecal starch was greater for BOV than CON, suggesting that Bovamine® actually reduced total tract starch digestibility (Table 12), particularly following the switch to the high starch ration (Figure 13). This is the opposite of what was expected, as Bovamine® was hypothesized to improve digestive function and stability. While others have not reported decreased starch digestibility, Raeth-Knight et al. (2007) and Boyd et al. (2011) did not observe an increase in starch digestibility in Bovamine® treated cows compared to unsupplemented cows.

Rumen and fecal bacterial microbiome

The only experimental factor that correlated with the bacterial composition of the samples was sample type, feces or rumen fluid, and Figure 14 shows a clear separation of samples by type. Multidimensional scaling (MDS) of the samples, using the weighted unifrac distance between their OTU count vectors, demonstrated two distinct clusters of microbial communities. Fecal samples were more closely clustered with each other than with rumen fluid samples, and vice versa, indicating differences in community structure between rumen and fecal samples. While it is expected that feces and rumen fluid have different bacterial compositions, this figure shows the efficacy of the microbial analysis since samples were clustered according to type. Figure 15 illustrates differences among samples for the 40 most prevalent families using a heatmap. Heatmaps are useful for visualizing abundance of individual families, as displayed by color, across treatments or, in this case, sample type. The color gradient assigns families of identified OTUs by abundance. Darker black indicates lower abundance of that family and the lighter blue indicates greater abundance. The MDS ordination in Figure 14 and the heatmap in Figure 15 do not show any additional clustering beyond sample type, indicating that effects of ration starch percentage and BOV treatment were minimal as further detailed below.

Figure 16 represents a heatmap of the 40 most prevalent OTU families of the processed rumen fluid samples. Those samples were filtered as described in the materials and methods above. The organisms are classified by family along the Y-axis, and the X-axis is arranged by treatment and ration starch percentage (for ease of

presentation the 23.8% starch ration is displayed as “20” and the 31.1% starch ration is displayed as “30”). Observing across the heatmap, there were no clear pattern differences when comparing treatment, suggesting a minimal effect of treatment on rumen fluid. MDS was also used to demonstrate rumen fluid microbiome differences between samples with regard to ration starch (color of marker) and CON or BOV treatment (shape of marker; Figure 17). The lack of organized clustering by treatment of dietary starch again demonstrates a lack of experimental effects on rumen fluid bacterial composition. Those families with at least 1% abundance in the rumen fluid samples are presented in Table 13. CON or BOV did not impact any of the most abundant families ($P = 1.00$). Although increased dietary starch numerically decreased *Prevotellacae*, increased family S24-7 within the order *Bacteroidales*, and decreased *Spirochaetaceae*, these differences were not significant ($P \geq 0.20$).

It is known that there are many factors than have an effect on the rumen microbial community. For example, changes in diet, age, use of antibiotics, geographic location, season, stress, and environment are only a few (Puniya et al., 2015). While all these factors may play a role in the rumen microbiome, the impact of diet has been most studied. For example, it is possible to manipulate the composition of ruminal bacteria by diet management. Crater et al. (2007) and Pulido et al. (2009) observed that microbial species and their activities were altered by adjusting feed intake and frequency of feeding. But the primary factor in inducing change in ruminal bacterial communities is diet transition (Tajima et al., 2001; Petri et al., 2013). Bacterial communities shift depending on available substrate. Starch and sugar degrading

microbes constitute the largest part of the ruminal bacterial population. These organisms are of great importance for high-producing ruminant animals since their diet usually contains large amounts of readily fermentable starch and sugars (Deusch, et al., 2017). Across all the rumen fluid samples in our study, over 70% of the rumen bacterial community was dominated by the Bacteroidetes phylum, followed by over 20% being from the phylum of Firmicutes. Deusch et al. (2017) and Petri et al. (2013) also found the top phylum to be Firmicutes followed by Bacteroidetes. In our study, the *Prevotellaceae* family was the most dominant bacterial family within the rumen ecosystem as reported before (Kim et al., 2011; Jami and Mizrahi, 2012; Deusch et al., 2017). Petri et al. (2013) also found *Prevotellaceae* to be most prominent in heifers fed 91% grain and concentrate diets. Members belonging to this family have versatile metabolic capabilities. They are able to utilize a broad range of substrates including peptides, proteins, monosaccharides, and plant polysaccharides (Miyazaki et al., 1997; Purushe et al., 2010). This family of organisms can be found in the rumen ecosystem across a variety of diets suggesting that these bacteria exhibit substantial metabolic diversity (Petri et al., 2013).

Although the changes were not significant, we observed an increase in abundance of organisms from the *Ruminococcaceae* family during the switch to the higher starch ration (~11% to ~17%). Petri et al. (2013) also observed the abundance of *Ruminococcacea* to be 18.09% in animals fed a mixed 60% forage and 91% grain and concentrate diet. Typically, when faced with a dietary transition, starch fermenters and fibrolytic species can change significantly, changes we did not observe in this

experiment. During the high starch ration diet, we expected to see the microbial population shift with decreasing cellulolytic bacteria, like *Ruminococcaceae,* and increasing acid tolerant bacteria like *Streprococcus* and *Lactobacillus* species. Khafipour et al. (2009) reported these changes when animals were given a high concentrate diet to induce SARA. Hungate et al. (1952) also reported that an excess of grain included in the ruminant diet will decrease cellulolytic bacteria and increase gram positive bacteria. Tajima et al. (2001) saw major decreases in *Fibrobacter succinogenes, Ruminococcus flavefaciens, Prevotella ruminicola, Ehrlichia ruminantium* and *Treponema bryantii* and increases in *Prevotella byrantii, Selenomonas ruminanrium* and *Mitsuokella multiacida* when switching animals from a hay to a grain diet. In opposition to this, numerically the abundance of organisms from the *Ruminococcaceae* family actually increased in our study when cows were fed a higher starch diet. But we did not observe any significant changes between treatments and diets. When Petri et al. (2013) compared overall diversity of their rumen bacterial samples, cluster analysis showed no significant clustering of profiles between animals, dietary treatment (forage, mixed forage, concentrate diets), or digesta matter. While dietary treatments in their study clustered separately from one another, there was no significant difference.

Figure 18 represents a heatmap of the 40 most prevalent OTU families of fecal samples. Organisms are classified by family along the Y-axis, with treatment (CON or BOV) and amount of starch (20 or 30) along the X-axis. Viewing across the heatmap, there were no clear pattern differences between treatments or ration starch percentage,

suggesting a minimal effect of treatment or dietary starch on feces. A lack of impact of ration or treatment on fecal microbiome is further illustrated by MDS in Figure 19. Those families with at least 1% abundance in the feces samples are presented in Table 14. Treatment did not impact any of the most abundant families ($P = 0.98$). Although increased dietary starch numerically decreased *Veillonellaceae*, the differences were not significant ($P = 0.12$).

Across all the fecal samples in our study, over 60% of the bacterial community was dominated by the Firmicutes phylum, followed by over 30% being from the phylum of Bacteroidetes. Deusch et al. (2017) found similar results in lactating Jersey cows where the top phylum in fecal samples was from the phylum Bacteroidetes followed by Firmicutes. It has been observed that the microbial population of the lower GIT in cattle are dominated by strict anaerobes such as *Bacteroides, Clostridium,* and *Bifidobacterium* species (Drasar and Barrow, 1985). The most abundant organisms found in our fecal samples came from the family of *Ruminococcaceae*. *Ruminococcaceae* break down complex carbohydrates and are common GIT microbes. In our study, there was no change in this family of organisms between diets or treatment. Shanks et al. (2011) suggested organisms from the family *Ruminococcaceae* can shift dramatically depending on diet. Organisms from the *Bacteroidales* order were found in the second highest abundance in our fecal samples. They are well known intestinal bacteria that can be both beneficial and harmful and are the most abundant gram-negative organisms in the human colonic microbiota (Mazmanian et al., 2005). The third most abundant organism we observed in our fecal microbiome samples come

from the order of Clostridiales. The broad genus *Clostridium* falls under this order, they are ubiquitous in the GIT and described as a "trash can" genus (Dowd et al., 2008). Organisms belonging to this classification can have both positive and negative effects on the host animal. *C. perfringes, C. tetani,* and *C. botulinum* are a few species that cause significant productivity problems (Reilly and Attwood, 1998). While others can improve digestion of cellulose and act as a beneficial probiotic (Kopecny et al., 1996).

The synergism between different groups of rumen microbial communities is so diverse and complicated, it is difficult to define a specific role for any particular group (Kamra, 2005). Rumen bacteria are not present in a single colony, they work in a symbiotic relationship with other rumen organisms forming a larger complex community. Studies using beef steers have shown that certain microbes may be associated with feed efficiency in cattle by improving ADG, DMI, feed conversion ratio, and residual feed intake (Guan et al., 2008; Hernandez-Sanabria et al., 2010). The effect of diet on rumen function was evaluated. In these studies, three bacterial species, *Succinivibrio, Eubacterium*, and *Robinsoniella* have been identified to correlate with feed efficiency measures. While the exact reason is not known, it is hypothesized that is has to do with their metabolic mechanisms including propionate synthesis, formate production, and cross-feeding interaction with methanogens (Hernandez-Sanabria et al., 2012). We did not observe these species with 1% or more abundance in the ruminal microbiome samples, likely because lactating dairy cattle diets differ significantly from feedlot diets. Being able to identify which microbial communities are related to efficiency in cattle can help the industry select for more efficient animals.

Chapter 5

CONCLUSIONS

The dairy industry is always trying to improve feed efficiency due to the high input of feed costs. One potential method to increase feed efficiency is to supplement a DFM into dairy cow diets. Experiment 1 evaluated the impact of feeding DFM Bovamine® to early lactation dairy cows on performance and nutrient digestibility. Overall, no benefits of Bovamine® on cow intake, milk yield, feed efficiency or milk composition were observed. BOV appeared to have improved starch digestion as demonstrated by a tendency for increased total tract starch digestibility and reduced fecal starch content during week 4, but the magnitude of the effects were small.

Experiment 2 evaluated the effects of supplementing Bovamine® to a 23.8% starch ration as well as during an abrupt transition to 31.1% starch ration. Independent of treatment, feeding the higher starch ration increased rumen VFA but did not affect performance variables or rumen pH. The lack of effects on rumen pH were surprising and suggest that there was sufficient rumen buffering to avoid a decrease in rumen pH with the increased VFA load. During the transition to the high starch ration, Bovamine® did not affect milk production or DMI. Bovamine® did increase milk fat yield which was largely driven by differences observed in primiparous cows and on day 1 of the transition to the high starch ration. Counter to the results of Experiment 1, in Experiment 2 Bovamine® actually increased fecal starch, suggesting it reduced total

tract starch digestibility. No effects of Bovamine® treatment or ration starch percentage on rumen and fecal microbiomes were observed. The lack of impact of the ration starch percentage on rumen and fecal microbiome was surprising but is supported by the lack of change in rumen pH and fecal measures. Future work may be improved by adding additional animals to improve power and by using a more fermentable starch source than corn grain to induce a more dramatic digestive disturbance.

TABLES

Table 1. Ingredient composition of Experiment 1 ration. Ingredients are expressed as a percentage of total ration dry matter.

Ingredient	% of ration DM
Corn silage	51.47
Alfalfa silage	8.90
Alfalfa hay	8.58
Ground corn	8.02
Protected soybean meal[1]	6.92
Canola meal	5.42
Citrus pulp	2.34
Sugar byproduct[2]	1.67
Porcine blood meal	1.64
Rumen bypass fat[3]	1.39
Sodium bicarbonate	0.73
Corn gluten meal	0.54
Trace mineral and vitamin mix[4]	0.46
Sodium chloride	0.37
Calcium carbonate	0.32
Potassium carbonate[5]	0.30
Monensin[6]	0.29
Monocalcium phosphate	0.28
Methionine precursor[7]	0.083
Potassium and magnesium sulfate[8]	0.061
Rumen protected methionine[9]	0.053
Urea	0.049
Rumen protected lysine[10]	0.042
Vitamin E, 46 KIU/kg	0.034
Magnesium oxide	0.023
Chelated zinc[11]	0.008
Biotin[12]	0.004

[1]Extruded and expelled soybean meal (J. L. Moyer & Sons, Inc., Turbotville, PA).
[2]Contained 92.3% sucrose (Renaissance Nutrition Inc., Roaring Spring, PA).
[3]MEGALAC (Church & Dwight Co., Inc, Princeton, NJ).
[4]Contained 14.7% calcium, 34.3% magnesium, 0.75% sulfur, 102 mg/kg Fe, 4,262 mg/kg Zn, 823 mg/kg Cu, 4,215 mg/kg Mn, 65.5 mg/kg Se, 141 mg/kg Co, 191 mg/kg I, 191 mg/kg I, 1,268 KIU/kg Vitamin A, 254 KIU/kg Vitamin D, and 5,062 IU/kg Vitamin E.
[5]DCAD Plus (Church & Dwight Co., Inc, Princeton, NJ).
[6]Rumensin 90 (Elanco, Greenfield, IN).
[7]HMTBa (MFP, Novus International, Inc., St. Charles, MO).
[8]Dynamate (18% K, 11% Mg, 22% S; The Mosaic Company, Plymouth, MN).
[9]Smartamine M (Adisseo, Antony, France)
[10]AjiPro-L Generation 2 (Ajinomoto Heartland, Inc., Chicago, IL).
[11]MINTREX Zn (Novus International, Inc., St. Charles, MO).
[12]Microvit H Promix Biotin 2% (Adisseo, Anthony, France)

Table 2. Nutrient composition of total mixed ration in Experiment 1.

	Formulated	Analyzed
CP	16.7	16.2 ± 0.2
NDF	30.2	32.3 ± 1.7
ADF	19.4	21.8 ± 1.2
Starch	25.0	23.8 ± 2.1
NFC	42.1	45.0 ± 1.0
NE_L, Mcal/kg	1.73	1.69 ± 0.02
Ash	7.6	7.0 ± 0.6
Ca, % DM	0.85	0.98 ± 0.05
P, % DM	0.38	0.38 ± 0.01
Mg, % DM	0.37	0.40 ± 0.03
K, % DM	1.45	1.37 ± 0.11
Na, % DM	0.37	0.43 ± 0.05
Fe, mg/kg	271	344 ± 29
Mn, mg/kg	47	76 ± 12
Zn, mg/kg	66	73 ± 14
Cu, mg/kg	11	17 ± 7

Table 3. Ingredient composition of rations fed during Experiment 2 expressed as a percentage of total ration dry matter.

Ingredient	Weeks 1-5	Week 6
Corn silage	51.47	45.61
Alfalfa silage	8.90	7.89
Alfalfa hay	8.58	7.61
Ground corn	8.02	18.47
Protected soybean meal[1]	6.92	6.13
Canola meal	5.42	4.81
Citrus pulp	2.34	2.08
Sugar byproduct[2]	1.67	1.48
Porcine blood meal	1.64	1.45
Rumen bypass fat[3]	1.39	1.23
Sodium bicarbonate	0.73	0.65
Corn gluten meal	0.54	0.48
Trace mineral and vitamin mix[4]	0.46	0.41
Sodium chloride	0.37	0.33
Calcium carbonate	0.32	0.28
Potassium carbonate[5]	0.30	0.27
Monensin[6]	0.29	0.26
Monocalcium phosphate	0.28	0.25
Methionine precursor[7]	0.083	0.076
Potassium and magnesium sulfate[8]	0.061	0.053
Rumen protected methionine[9]	0.053	0.045
Urea	0.049	0.045
Rumen protected lysine[10]	0.042	0.038
Vitamin E, 46 KIU/kg	0.034	0.030
Magnesium oxide	0.023	0.023
Chelated zinc[11]	0.008	0.008
Biotin[12]	0.004	0.004

[1]Extruded and expelled soybean meal (J. L. Moyer & Sons, Inc., Turbotville, PA).
[2]Contained 92.3% sucrose (Renaissance Nutrition Inc., Roaring Spring, PA).
[3]MEGALAC (Church & Dwight Co., Inc, Princeton, NJ).
[4]Contained 14.7% calcium, 34.3% magnesium, 0.75% sulfur, 102 mg/kg Fe, 4,262 mg/kg Zn, 823 mg/kg Cu, 4,215 mg/kg Mn, 65.5 mg/kg Se, 141 mg/kg Co, 191 mg/kg I, 191 mg/kg I, 1,268 KIU/kg Vitamin A, 254 KIU/kg Vitamin D, and 5,062 IU/kg Vitamin E.
[5]DCAD Plus (Church & Dwight Co., Inc, Princeton, NJ).
[6]Rumensin 90 (Elanco, Greenfield, IN).
[7]HMTBa (MFP, Novus International, Inc., St. Charles, MO).
[8]Dynamate (18% K, 11% Mg, 22% S; The Mosaic Company, Plymouth, MN).
[9]Smartamine M (Adisseo, Antony, France)
[10]AjiPro-L Generation 2 (Ajinomoto Heartland, Inc., Chicago, IL).
[11]MINTREX Zn (Novus International, Inc., St. Charles, MO).
[12]Microvit H Promix Biotin 2% (Adisseo, Anthony, France)

Table 4. Nutrient composition of total mixed ration of Experiment 2.

	Week 1-5		Week 6	
	Formulated	Analyzed	Formulated	Analyzed
CP	16.7	16.2 ± 0.2	15.8	15.5 ± 0.2
NDF	30.2	32.3 ± 1.7	27.9	28.6 ± 0.3
ADF	19.4	21.8 ± 1.2	17.7	19.0 ± 0.1
Starch	25.0	23.8 ± 2.1	30.5	31.1 ± 0.3
Ash	7.6	7.0 ± 0.6	7.0	6.8 ± 0.6

Table 5. LSmeans of weekly intake, milk yield, and milk composition results from all cows over the course of Experiment 1.

	Treatment			*P* values			
	Control	Bovamine	SEM	Treatment	Week	Treatment × Week	Covariate
DMI, kg/d	27.1	26.0	0.5	0.16	0.002	0.09	0.01
Milk, kg/d	45.9	45.8	1.0	0.94	0.001	0.95	0.001
Fat, %	3.85	3.62	0.11	0.13	0.004	0.26	0.001
Fat, kg/d	1.75	1.67	0.04	0.22	0.16	0.47	0.001
Protein, %	2.92	2.89	0.04	0.54	0.001	0.38	0.001
Protein, kg/d	1.33	1.31	0.02	0.42	0.001	0.63	0.001
ECM, kg/d	47.7	46.7	0.8	0.37	0.001	0.74	0.001
3.5FCM, kg/d	48.0	46.9	0.8	0.36	0.001	0.65	0.001
Milk/DMI, kg/kg	1.72	1.75	0.04	0.55	0.001	0.14	0.001
ECM/DMI, kg/kg	1.79	1.78	0.04	0.96	0.001	0.50	0.04
3.5FCM/DMI, kg/kg	1.80	1.79	0.05	0.87	0.001	0.45	0.02
MUN, mg/dL	12.1	11.8	0.3	0.48	0.001	0.43	0.002
SCS	2.42	2.08	0.18	0.21	0.007	0.37	0.001
BW, kg[1]	729	723	6	0.52	0.001	0.61	0.001

[1]Body weight (BW) was measured weeks 5 and 10 only.

Table 6. LSmeans of weekly intake, milk yield, and milk composition results from over the course of the experiment. Cows with chronically high SCC (47, 51, 110, 953, and 997) and outlier cow 6 were excluded from analyses.

	Treatment			*P* values			
	Control	Bovamine	SEM	Treatment	Week	Treatment × Week	Covariate
DMI, kg/d	26.6	25.9	0.4	0.21	0.001	0.01	0.001
Milk, kg/d	45.3	45.5	0.8	0.92	0.001	0.54	0.001
Fat, %	3.90	3.80	0.09	0.43	0.001	0.03	0.001
Fat, kg/d	1.75	1.74	0.05	0.98	0.21	0.32	0.001
Protein, %	2.94	2.95	0.03	0.87	0.001	0.38	0.001
Protein, kg/d	1.32	1.33	0.02	0.97	0.001	0.52	0.001
ECM, kg/d	47.5	47.7	0.9	0.88	0.001	0.77	0.001
3.5FCM, kg/d	47.8	48.0	1.0	0.93	0.001	0.63	0.001
Milk/DMI, kg/kg	1.71	1.75	0.03	0.46	0.001	0.06	0.001
ECM/DMI, kg/kg	1.81	1.82	0.04	0.85	0.001	0.25	0.001
3.5FCM/DMI, kg/kg	1.82	1.83	0.04	0.89	0.001	0.19	0.001
MUN, mg/dL	12.2	11.8	0.3	0.40	0.001	0.28	0.008
SCS	1.75	1.53	0.19	0.45	0.02	0.20	0.001
BW, kg[1]	719	721	6	0.81	0.001	0.96	0.001

[1]Body weight (BW) was measured weeks 5 and 10 only.

Table 7. LSmeans of fecal score and fecal starch collected at weeks 1, 2, 4, 6, 8, and 10 from all cows during Experiment 1. LSmeans of apparent total tract nutrient digestibility evaluated from a subset of 14 cows (7 per treatment) at weeks 1, 2, 4, 6, 8, and 10.

	Treatment			*P* values			
	Control	Bovamine	SEM	Treatment	Week	Treatment × Week	Covariate
Fecal score	2.96	3.07	0.07	0.23	0.001	0.41	0.001
Fecal starch, %	0.78	0.59	0.12	0.30	0.001	0.01	0.73
DM Digestibility, %							
DM	68.8	69.2	0.3	0.42	0.001	0.44	0.58
OM	70.2	70.5	0.3	0.47	0.001	0.46	0.62
Starch	98.46	98.74	0.09	0.051	0.002	0.13	0.97
CP	69.2	69.3	0.4	0.92	0.001	0.27	0.21
NDF	42.0	42.7	0.7	0.53	0.001	0.45	0.81
ADF	39.7	40.5	0.7	0.46	0.001	0.25	0.75

Table 8. LSmeans of weekly intake, milk yield, and milk composition results over the course of Experiment 2. Results of low producing cow 138 were excluded.

	Treatment			*P* values			
	Control	Bovamine	SEM	Treatment	Week	Treatment × Week	Treatment × Parity
DMI, kg/d	25.1	25.0	0.4	0.99	0.12	0.84	0.90
Milk, kg/d	38.1	39.3	2.9	0.35	0.55	0.88	0.54
Milk/DMI, kg/kg	1.53	1.57	0.09	0.23	0.002	0.75	0.25
Fat, %	3.40	3.47	0.20	0.49	0.97	0.74	0.60
Fat, kg/d	1.30	1.36	0.13	0.13	0.53	0.60	0.27
Protein, %	2.89	2.87	0.06	0.42	0.04	0.69	0.34
Protein, kg/d	1.10	1.12	0.07	0.17	0.94	0.61	0.06
Lactose, %	4.81	4.86	0.05	0.08	0.20	0.12	0.48
MUN, mg/dL	10.8	10.4	0.3	0.25	0.03	0.90	0.88
SCS	1.76	1.67	0.45	0.75	0.26	0.68	0.56

Table 9. LSmeans of intake, milk yield, and milk composition from day 7 of week 5 (normal starch ration) and days 1, 3, and 7 of week 6 (high starch ration) of Experiment 2. Results are excluding low producing cow 138.

	Treatment			*P* values			
	Control	Bovamine	SEM	Treatment	Day	Treatment × Day	Treatment × Parity
DMI, kg/d	24.9	25.7	0.6	0.30	0.81	0.13	0.26
Milk, kg/d	37.1	38.6	2.4	0.38	0.24	0.41	0.45
Milk/DMI, kg/kg	1.50	1.50	0.07	0.89	0.72	0.06	0.76
Fat, %	3.42	3.59	0.29	0.09	0.20	0.10	0.43
Fat, kg/d	1.28	1.39	0.15	0.002	0.04	0.02	0.05
Protein, %	2.93	2.88	0.04	0.27	0.22	0.52	0.60
Protein, kg/d	1.09	1.10	0.06	0.65	0.71	0.26	0.64
Lactose, %	4.86	4.87	0.05	0.64	0.05	0.54	0.28
MUN, mg/dL	9.47	9.71	0.22	0.28	0.004	0.69	0.53
SCS	1.74	1.83	0.36	0.80	0.21	0.94	0.73

Table 10. LSmeans of daily pH data collected during weeks 5 (normal starch ration) and 6 (high starch ration) of each period in Experiment 2. Results of all 6 cows are included.

	Treatment			*P* values				
	Control	Bovamine	SEM	Treatment	Week	Treatment × Week	Treatment × Day	Treatment × Day × Week
Mean pH	6.31	6.33	0.07	0.69	0.07	0.24	0.94	0.73
Minimum pH	5.85	5.87	0.06	0.22	0.05	0.20	0.72	0.76
Maximum pH	6.84	6.84	0.06	0.75	0.20	0.75	0.80	0.86
Minutes/d below pH 5.8	53	38	34	0.69	0.13	0.65	0.93	0.95
Area/d below pH 5.8	6.1	6.7	4.6	0.46	0.10	0.46	0.96	0.97

Table 11. LSmeans of rumen VFA and lactate measured 4, 10, 16, and 22 h after feeding on day 7 of week 5 and days 1 and 7 of week 6 in Experiment 2. LSmeans of in situ digestibility after 6, 12, 18, and 24 h in the rumen measured on day 7 of week 5 and days 1 and 7 of week 6. Results of all 6 cows are included.

	Treatment			*P* values					
	Control	Bovamine	SEM	Treatment	Day	Hour	Treatment × Hour	Treatment × Day	Treatment × Day × Hour
Organic acid, mM									
Acetate	65.0	65.2	1.8	0.94	0.001	0.001	0.89	0.42	0.46
Propionate	23.2	23.8	2.4	0.76	0.002	0.001	0.97	0.53	0.87
Isobutyrate	1.21	1.23	0.04	0.67	0.005	0.002	0.86	0.44	0.84
Butyrate	11.6	11.7	0.7	0.98	0.001	0.001	0.74	0.80	0.88
Isovalerate	1.63	1.79	0.14	0.11	0.01	0.001	0.52	0.10	0.94
Valerate	1.53	1.47	0.11	0.46	0.001	0.001	0.99	0.93	0.60
Total VFA[1]	105.5	107.0	4.7	0.73	0.001	0.001	1.00	0.37	0.89
Lactate[2]	0.50	0.53	0.12	0.90	0.39	0.002	0.37	0.77	0.99
Total organic acids[3]	105.9	107.5	4.7	0.72	0.001	0.001	1.00	0.38	0.90
Acetate to propionate ratio	2.97	3.00	0.24	0.88	0.57	0.001	0.79	0.29	0.70
In situ disappearance, % of starting	35.8	36.4	2.2	0.84	0.001	0.001	0.81	0.59	0.34

[1]Total VFA = sum of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate

[2]Lactate was log transformed prior to statistical analyses. LSmeans and SEM presented in the table were reverse transformed.

[3]Total organic acids = total VFA + lactate.

Table 12. LSmeans of fecal pH and fecal starch content. Fecal samples were collected at 0, 6, 12, and 18 h relative to feeding on day 7 of week 5, day 1 of week 6, and day 7 of week 6 in Experiment 2. Fecal pH was measured at each time. For starch analysis, fecal samples were composited by cow for each day. Results are from all 6 cows.

	Treatment			*P* values					
	Control	Bovamine	SEM	Treatment	Day	Hour	Treatment × Hour	Treatment × Day	Treatment × Day × Hour
Fecal pH	6.82	6.83	0.04	0.92	0.002	0.001	0.70	0.54	0.89
Starch, % DM	2.03	2.49	0.19	0.02	0.01	--	--	0.53	--

Table 13. Experiment 2. Percentage of organisms with at least 1% abundance found in rumen fluid for both treatments (Bov vs. Con) and both rations (23.8% vs. 31.1% starch). Adjusted *P* values assess the overall impact of treatment and ration starch percentage.

Organism	Treatment				Adj. *P*-values	
	CON 23.8%	BOV 23.8%	CON 31.1%	BOV 31.1%	Treatment	% Starch
f__Prevotellaceae	60.98%	60.43%	49.30%	45.94%	1.00	0.21
f__Ruminococcaceae	11.59%	11.11%	16.50%	18.93%	1.00	0.86
o_Bacteroidales f__unknown	5.99%	4.59%	6.62%	5.80%	1.00	1.00
f__[Paraprevotellaceae]	3.34%	3.27%	3.46%	3.32%	1.00	0.75
o_Clostridiales f__unknown	2.53%	2.32%	3.77%	4.20%	1.00	0.63
f__Lachnospiraceae	2.26%	2.97%	4.11%	3.90%	1.00	1.00
f__Veillonellaceae	2.20%	2.01%	1.91%	2.21%	1.00	0.75
o_Bacteroidales f__S24-7	1.90%	2.16%	4.05%	4.13%	1.00	0.20
c_Mollicutes o_RF39 f__unknown	1.79%	2.28%	2.71%	4.23%	1.00	0.60
f__Spirochaetaceae	1.48%	1.06%	0.77%	0.46%	1.00	0.20
o_Bacteroidales f__BS11	1.40%	2.41%	1.14%	1.08%	1.00	0.63
f__Succinivibrionaceae	1.33%	1.06%	0.66%	0.87%	1.00	0.47
o_Bacteroidales f__RF16	1.05%	1.09%	1.22%	1.07%	1.00	1.00

Table 14. Experiment 2. Percentage of organisms with at least 1% abundance found in feces for both treatments (Bov vs. Con) and both rations (23.8% vs. 31.1% starch). Adjusted *P* values assess the overall impact of treatment and ration starch percentage.

Organism	Treatment				Adj. *P*-values	
	CON 23.8%	BOV 23.8%	CON 31.1%	BOV 31.1%	Treatment	% Starch
f__Ruminococcaceae	33.84%	33.70%	36.03%	34.81%	0.98	0.98
o_Bacteroidales f__unknown	11.57%	12.25%	13.83%	13.68%	0.98	0.98
o_Clostridiales f__unknown	10.28%	6.86%	5.80%	4.92%	0.98	0.41
f__Bacteroidaceae	8.35%	9.49%	8.19%	10.20%	0.98	0.98
f__Rikenellaceae	6.62%	5.03%	7.74%	7.49%	0.98	0.78
c_Mollicutes o_RF39 f__unknown	4.61%	3.64%	2.57%	2.48%	0.98	0.78
f__Lachnospiraceae	4.60%	3.72%	3.71%	5.06%	0.98	0.98
f__[Paraprevotellaceae]	3.89%	4.93%	4.72%	5.37%	0.98	0.98
o_Bacteroidales f__S24-7	3.10%	5.51%	4.24%	3.17%	0.98	0.98
f__Veillonellaceae	2.22%	1.92%	0.71%	0.60%	0.98	0.12
o_Bacteroidales f__RF16	1.32%	1.28%	2.45%	2.01%	0.98	0.78
f__Bifidobacteriaceae	1.29%	3.32%	0.47%	0.58%	0.98	0.41
f__Spirochaetaceae	1.14%	0.88%	2.02%	1.81%	0.98	0.60

FIGURES

Figure 1. Number of reads for each Experiment 2 microbiome sample. X-axis shows the number of sequences read per sample and the Y-axis representing the number of samples (n = 72).

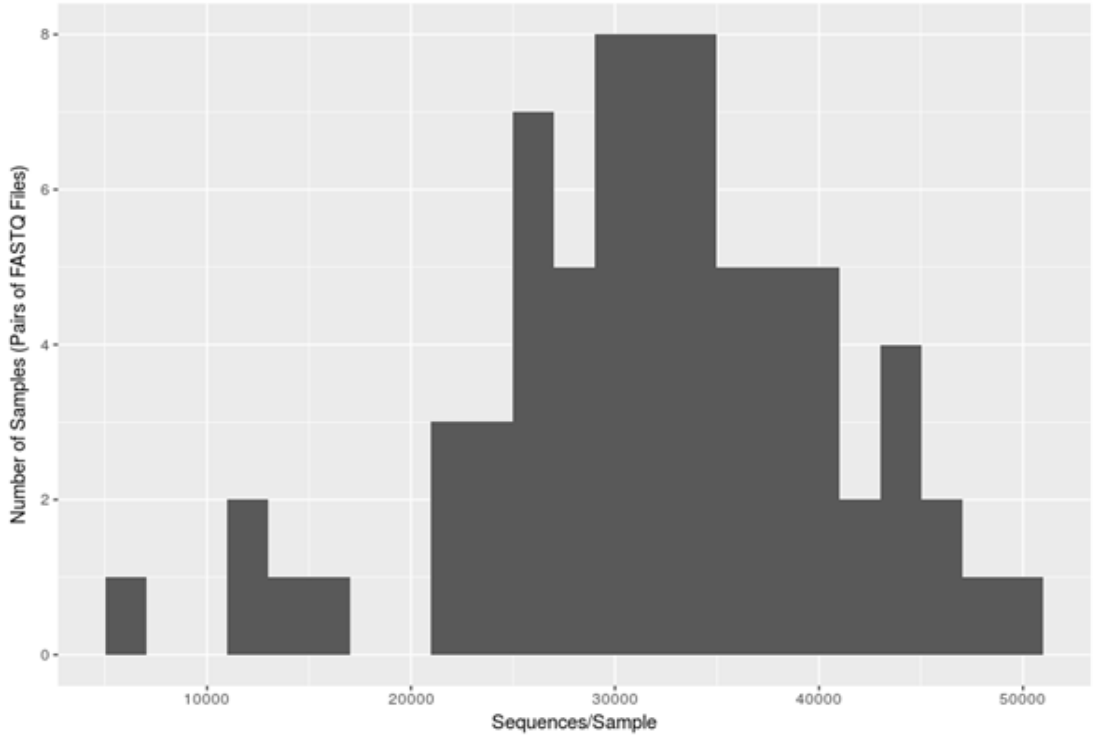


Figure 2. Quality histogram of the raw reads from the applications FastQC and MultiQC. Most of the nucleotide Phred quality scores were over 30. The reads with higher final qualities (~37) are the forward reads. The lower final qualities (~29) are the reverse reads. The first 20 nucelotides are of lower quality corresponding to the 515F primer.

Figure 3. Total number of OTU features per sample vs. the number of reads per sample. Most of the samples retained ~50% of the reads after running through DADA2. The cumulative reads retained is on a percentage basis. The 3 points highlighted were randomly selected to represent reads with poor, intermediate, and good retention after filtering.

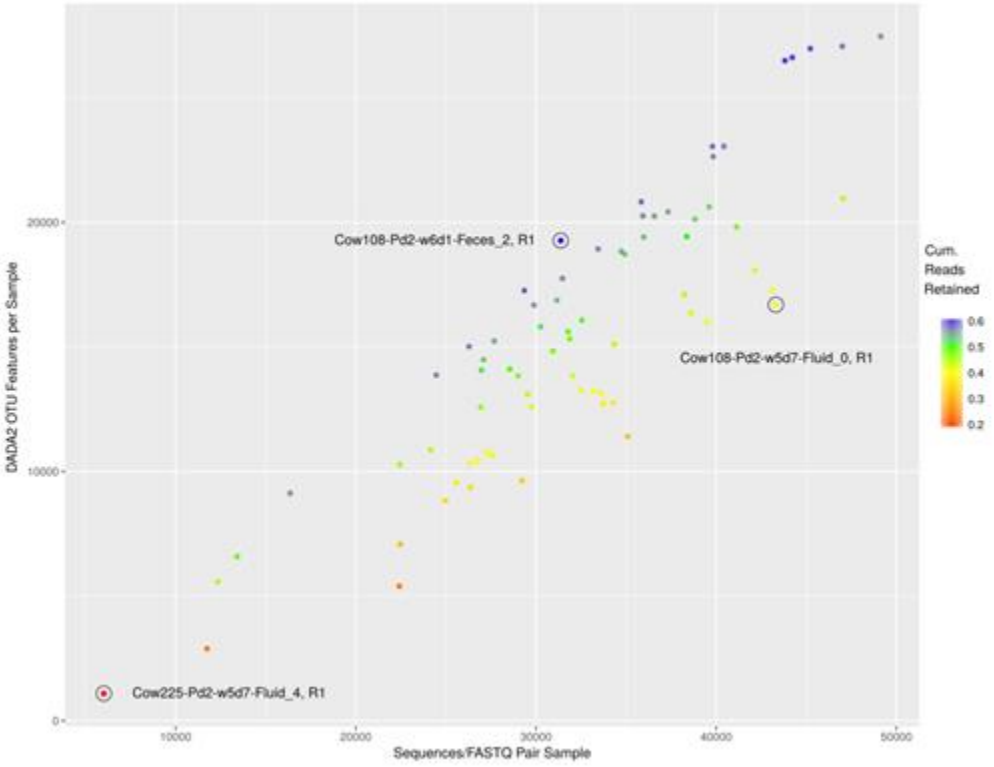


Figure 4. Histogram representing the OTU counts after filtering. OTUs that appeared less than 3 times, left of the red dashed line, were filtered out (n=433). These were considered to be noise.

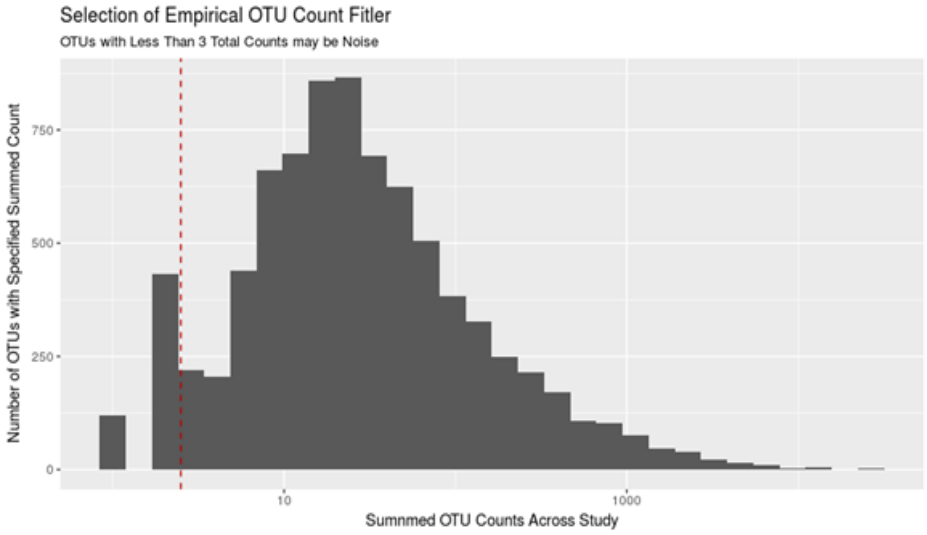


Figure 5. The naïve Bayes classifier was run with a confidence cutoff at 0.9. The mean confidence was 0.918, with a sharp peak at 0.99 to 1.0.

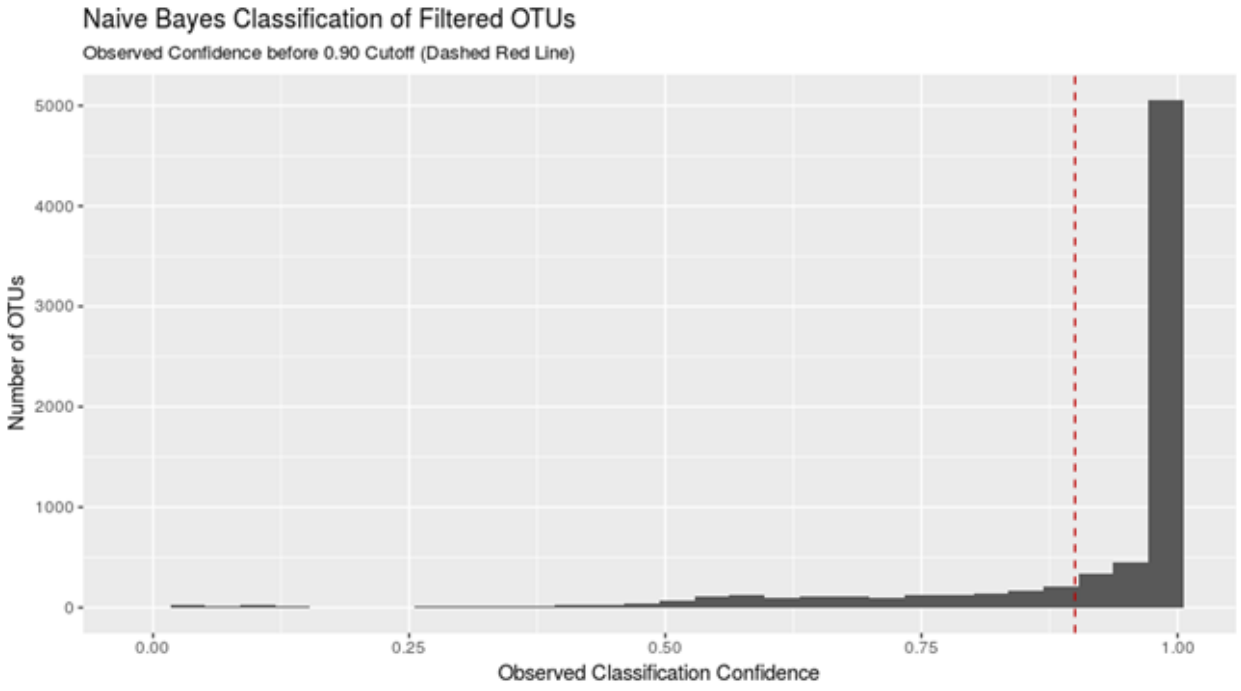


Figure 6. Taxonomic classification of microorganisms found in the filtered samples in decreasing order.

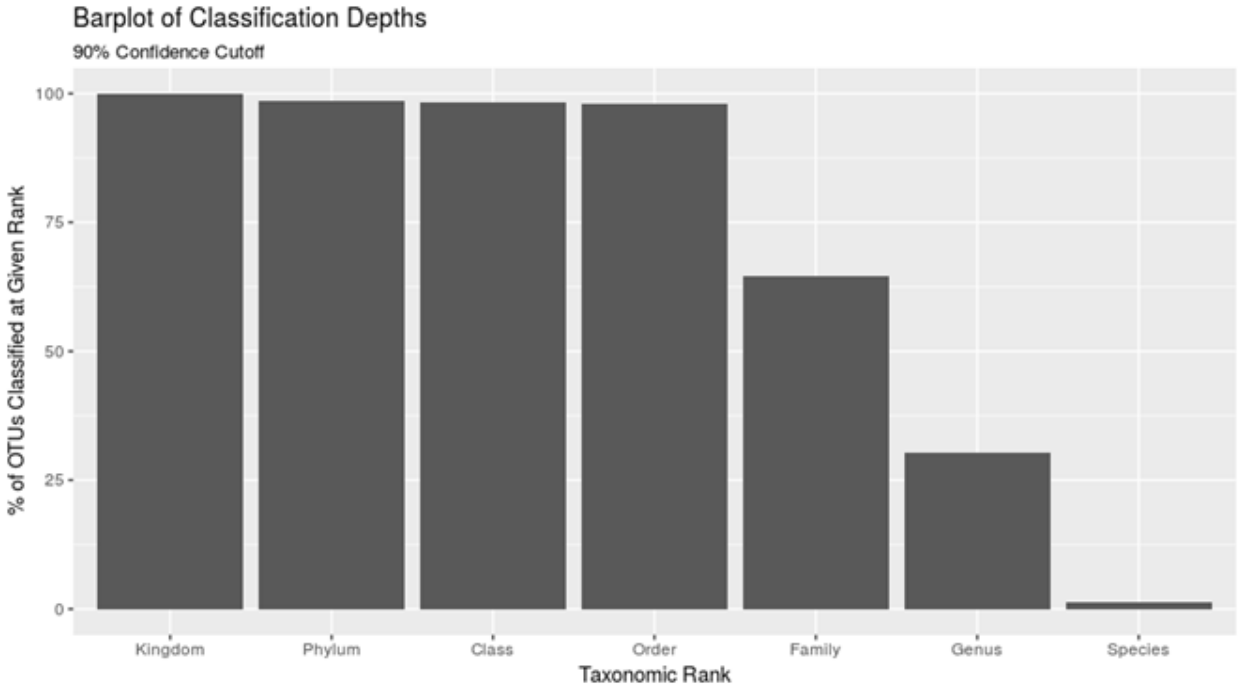


Figure 7. Experiment 1. Interaction of treatment by week ($P = 0.01$) for dry matter intake using weekly data but excluding cow 6 and cows with chronic high SCC (cows 47, 51, 110, 953, and 997). There tended to be a difference at week 4 ($P = 0.07$) and there was a difference at week 5 ($P = 0.004$).

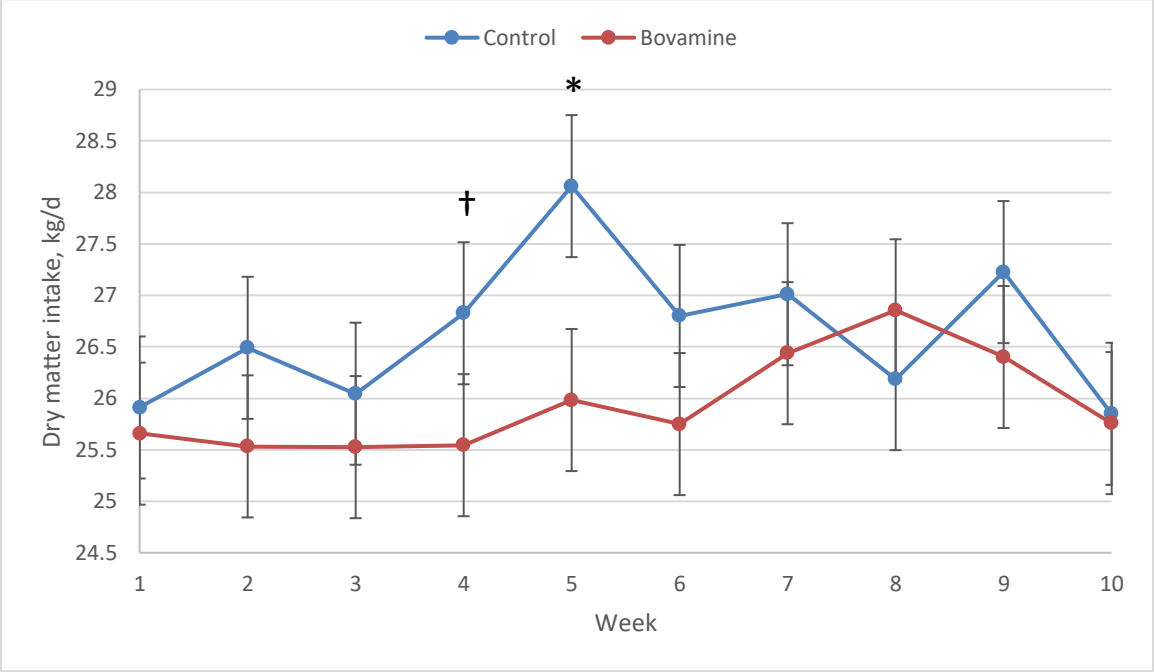


Figure 8. Experiment 1. Interaction of treatment by week ($P = 0.03$) for milk fat percentage using weekly data but excluding cow 6 and cows with chronic high SCC (cows 47, 51, 110, 953, and 997). There tended to be a difference at week 7 ($P = 0.07$).

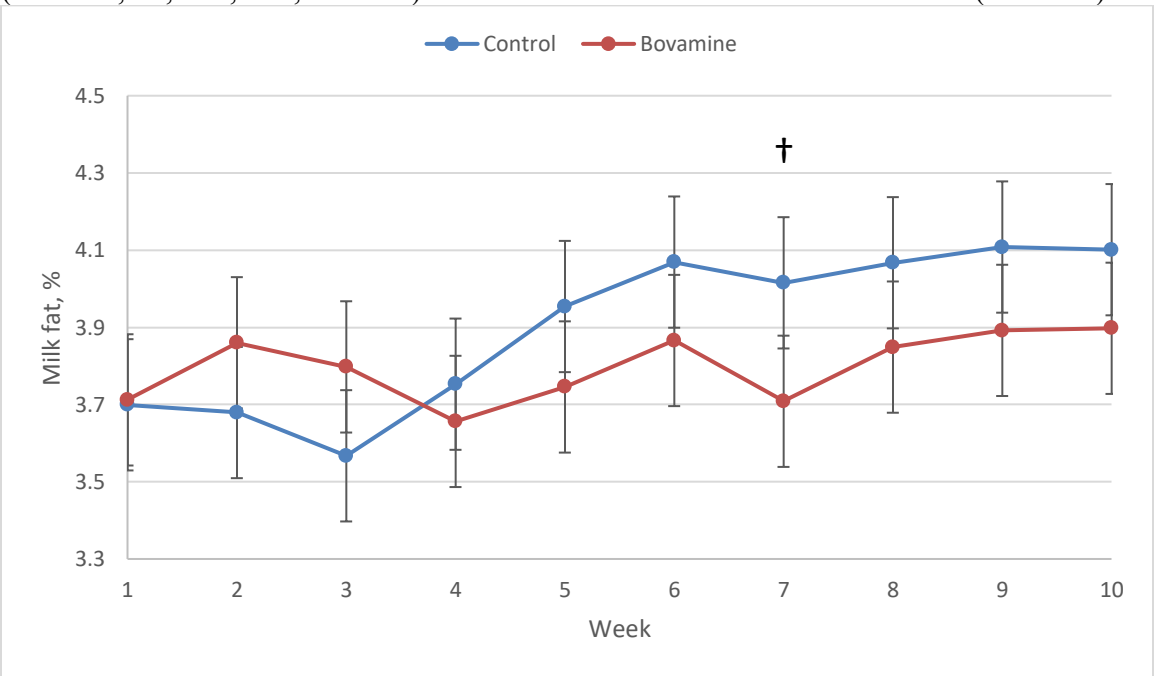


Figure 9. Experiment 1. Interaction of treatment by week ($P = 0.01$) for fecal starch. Fecal starch was greater for Control than Bovamine at week 2 ($P = 0.04$)

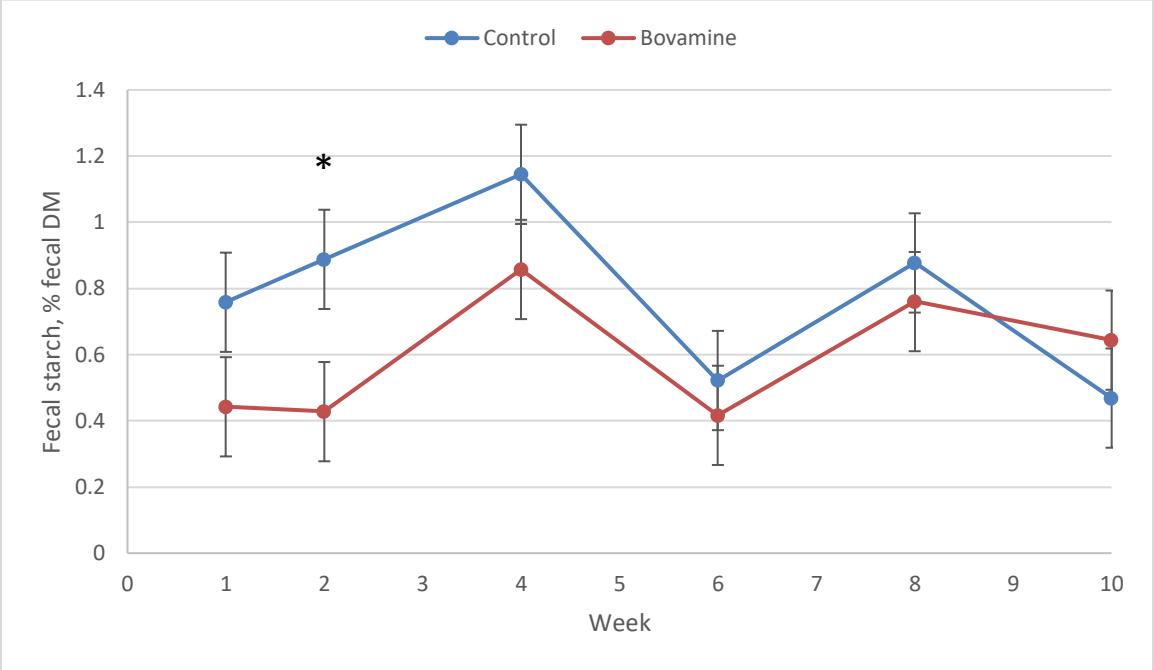


Figure 10. Experiment 2. Tendency for an interaction of treatment by parity ($P = 0.06$) for milk protein yield evaluated using weekly data but excluding cow 138.

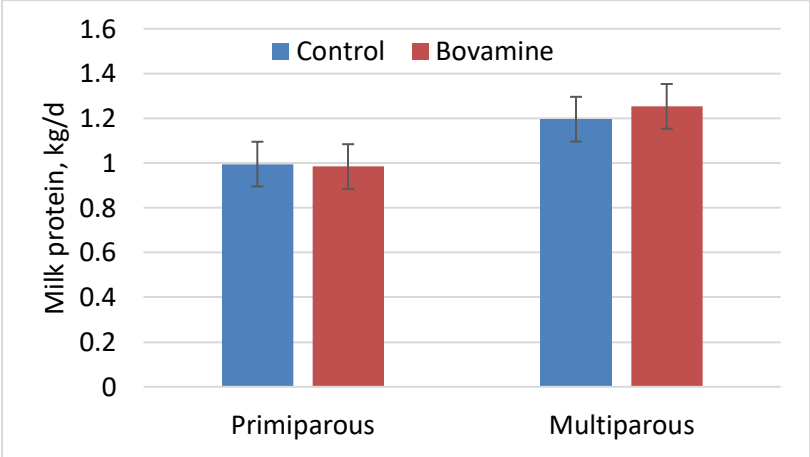
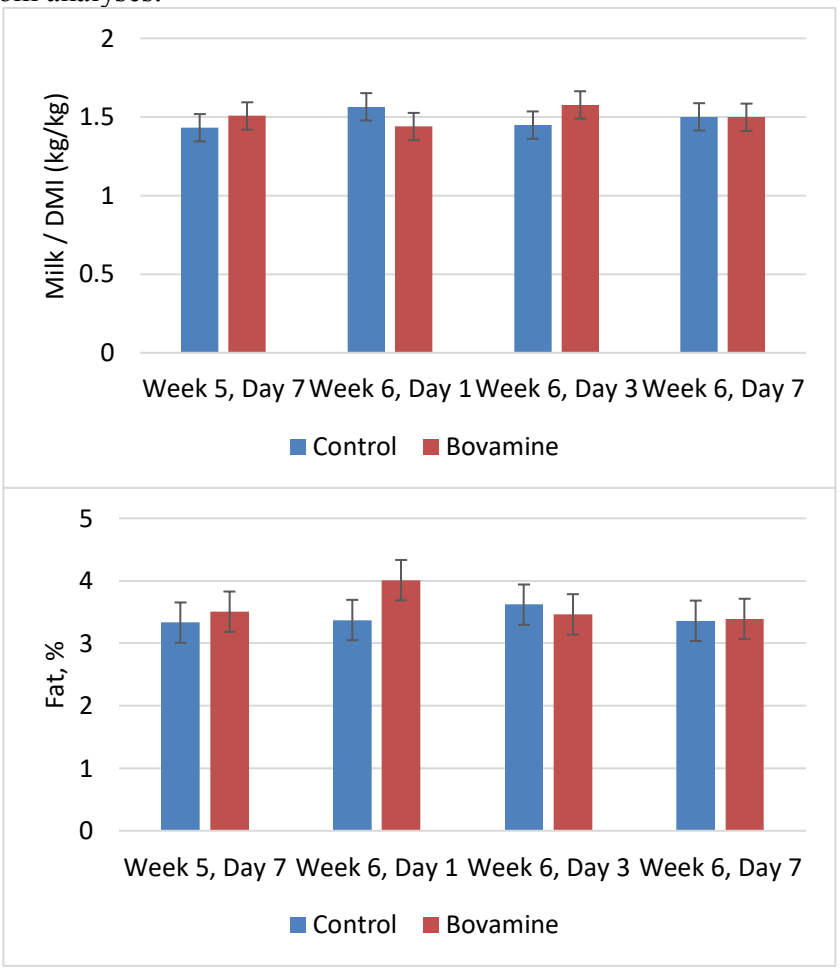


Figure 11. Experiment 2. Interactions of treatment by day for milk/DMI (P = 0.06), fat % (P = 0.10) and fat yield (P = 0.02) and interaction of treatment by parity for fat yield (P = 0.05) observed in the model evaluating results on day 7 of week 5 (normal starch ration) and days 1, 3, and 7 of week 6 (high starch ration). Low producing cow 138 was excluded from analyses.



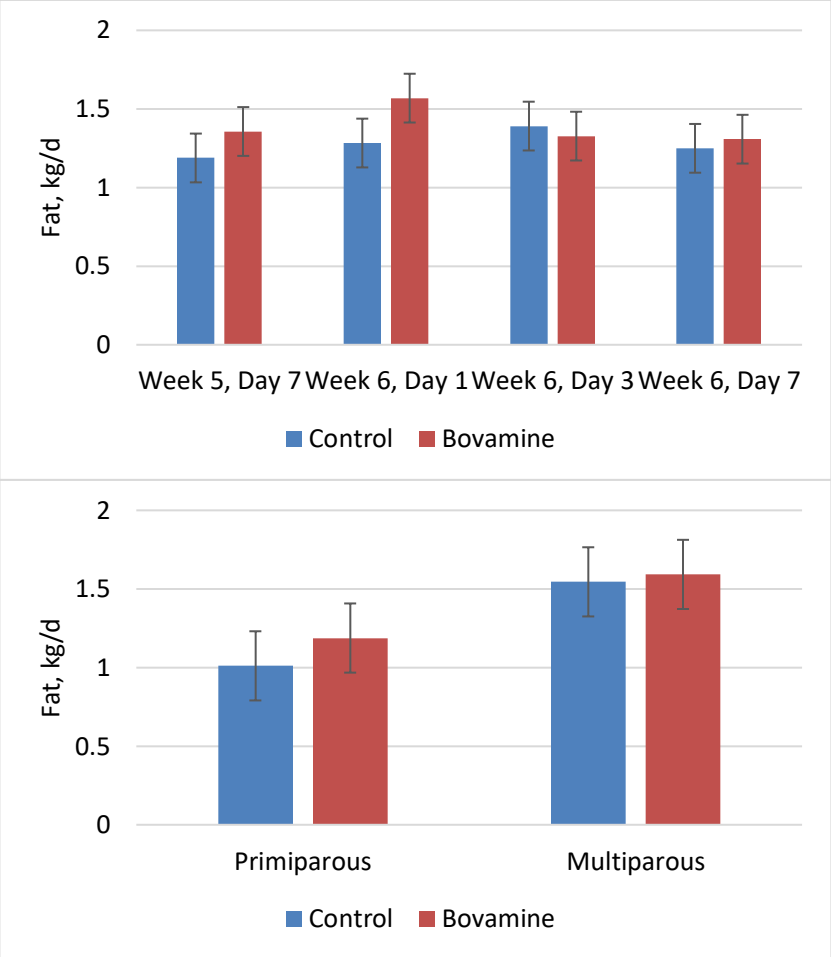

2
1.5
1
0.5
0
Fat, kg/d
Week 5, Day 7
Week 6, Day 1
Week 6, Day 3
Week 6, Day 7
Control
Bovamine
2
1.5
1
0.5
0
Fat, kg/d
Primiparous
Multiparous
Control
Bovamine

Figure 12a. Experiment 2. LSmeans of total rumen VFA evaluated on day 7 of week 5, day 1 of week 6, and day 7 of week 6 for all cows.

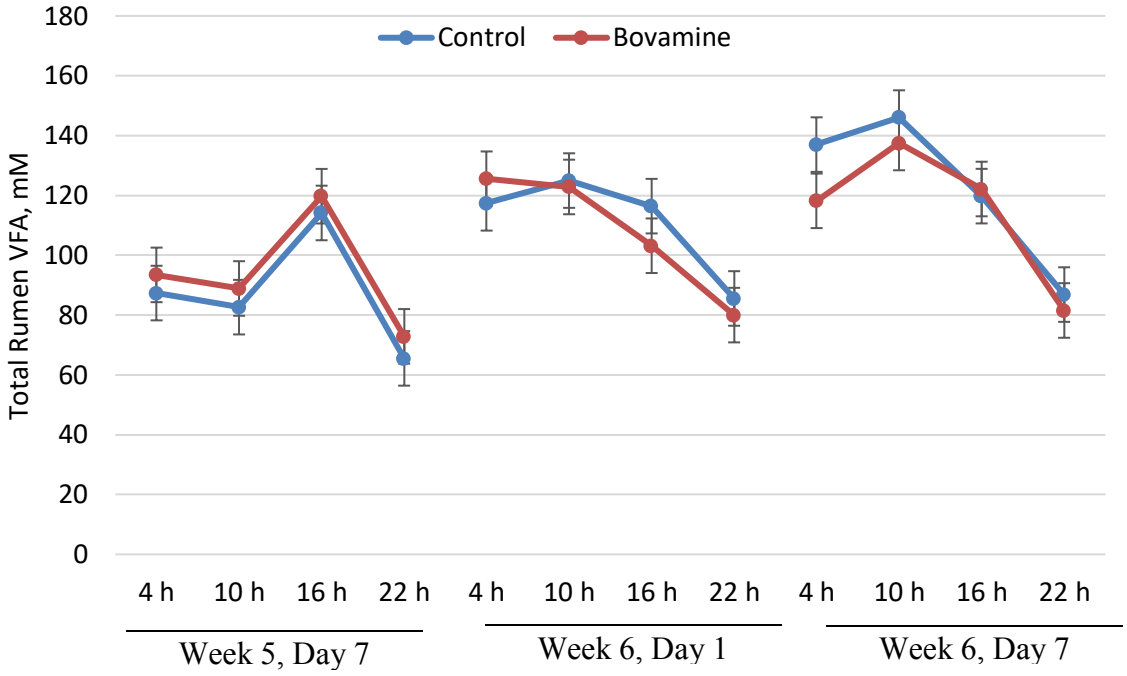


Figure 12b. Experiment 2. LSmeans of in situ digestibility evaluated on day 7 of week 5, day 1 of week 6, and day 7 of week 6 for all cows.

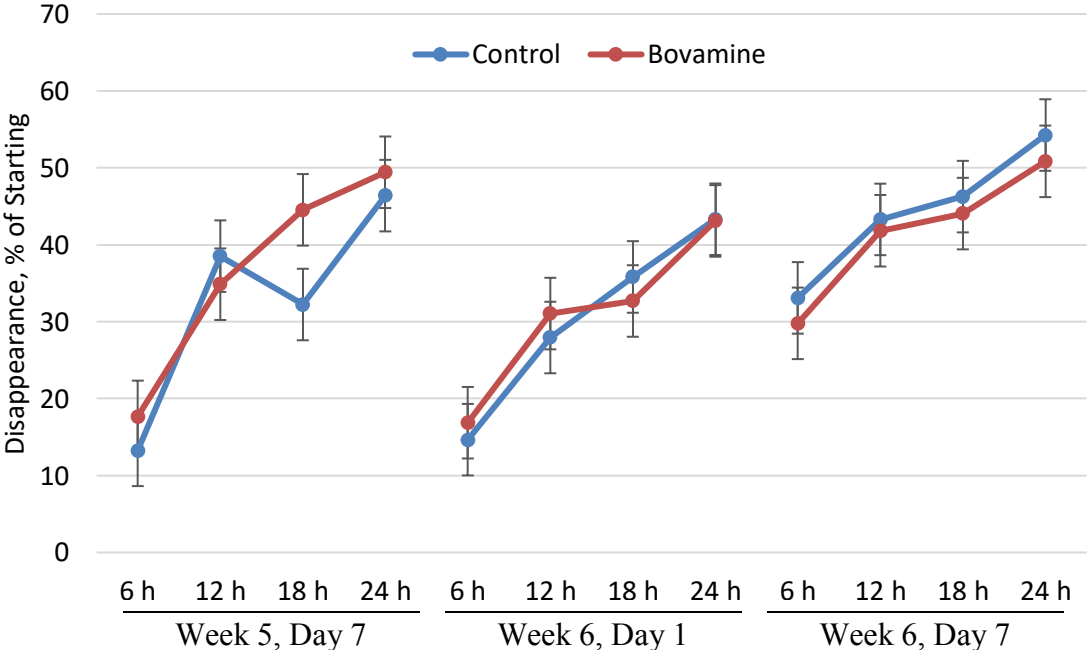


Figure 13. Experiment 2. Fecal starch observed on day 7 of week 5 (normal starch ration) and days 1 and 7 of week 6 (high starch ration). Fecal starch was affected by both treatment ($P = 0.02$) and day ($P = 0.01$), but there was no interaction of treatment by day ($P = 0.53$).

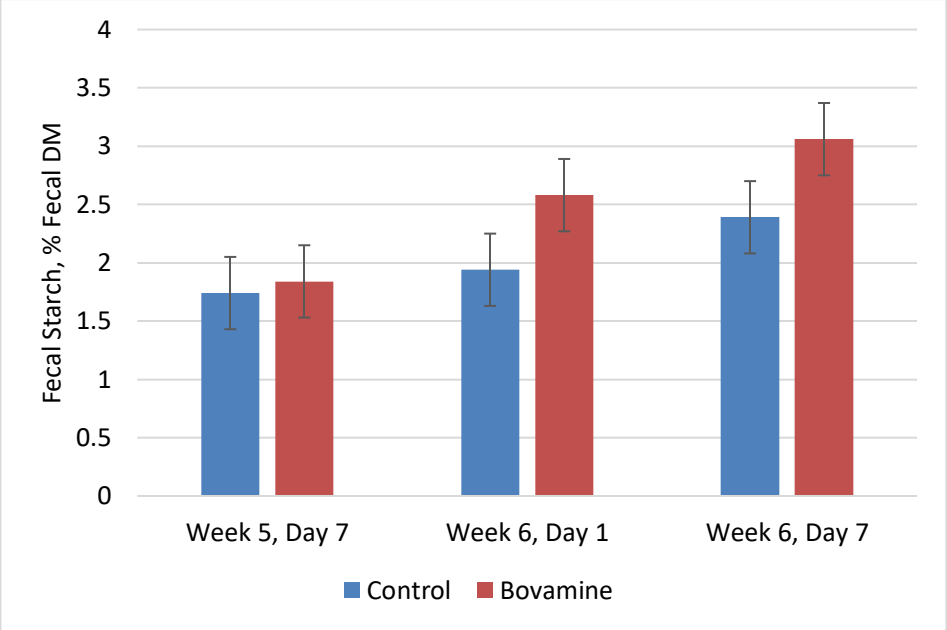


Figure 14. Experiment 2. Multidimensional scaling (MDS) of the filtered samples demonstrating two distinct clusters of microbial communities by sample type, rumen fluid or feces.

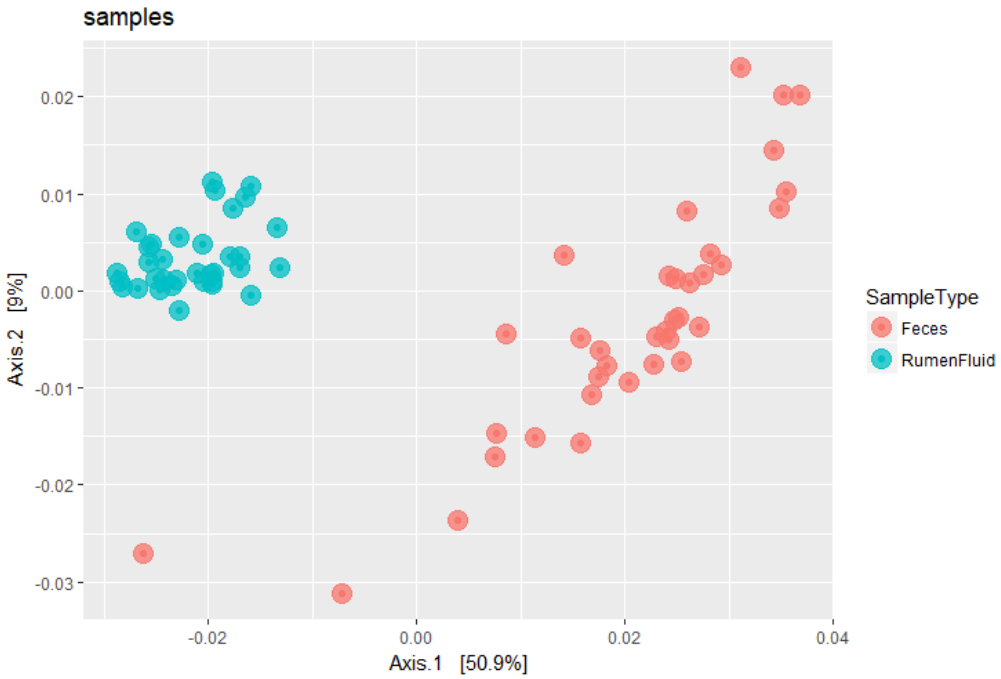


Figure 15. Experiment 2. Heatmap of the 40 most prevalent bacterial families found across all samples. Differences between rumen fluid and feces are evident.

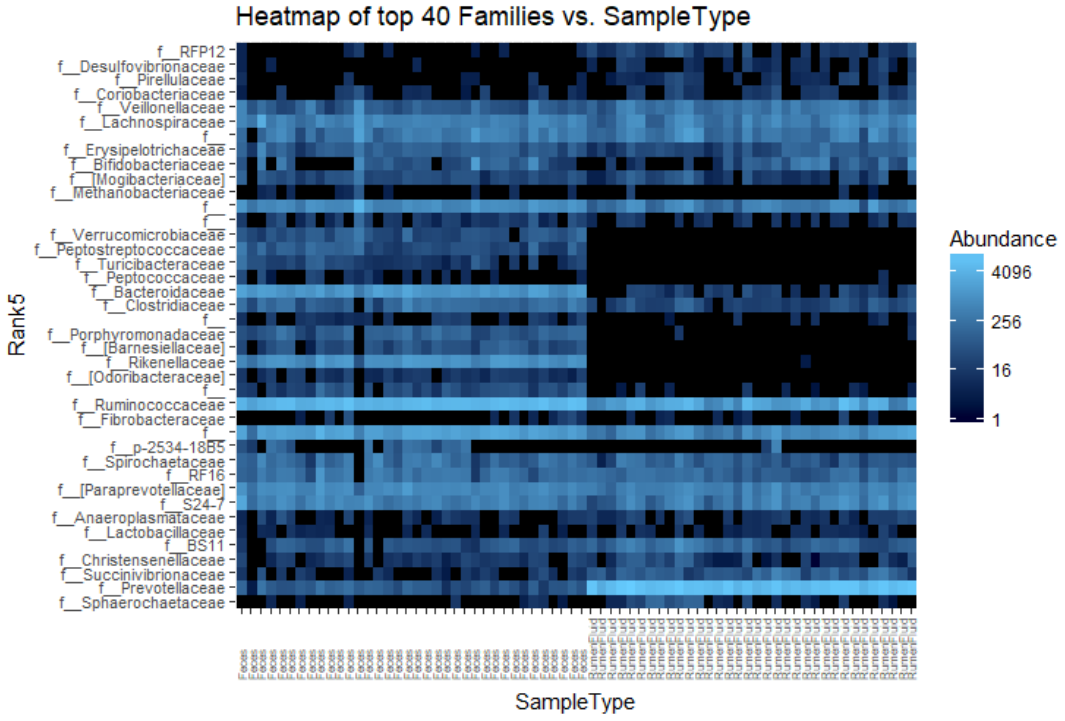


Figure 16. Experiment 2. Heatmap of bacteria families identified in rumen fluid samples collected during the low starch and the high starch ration by treatment (BOV vs. CON). For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".

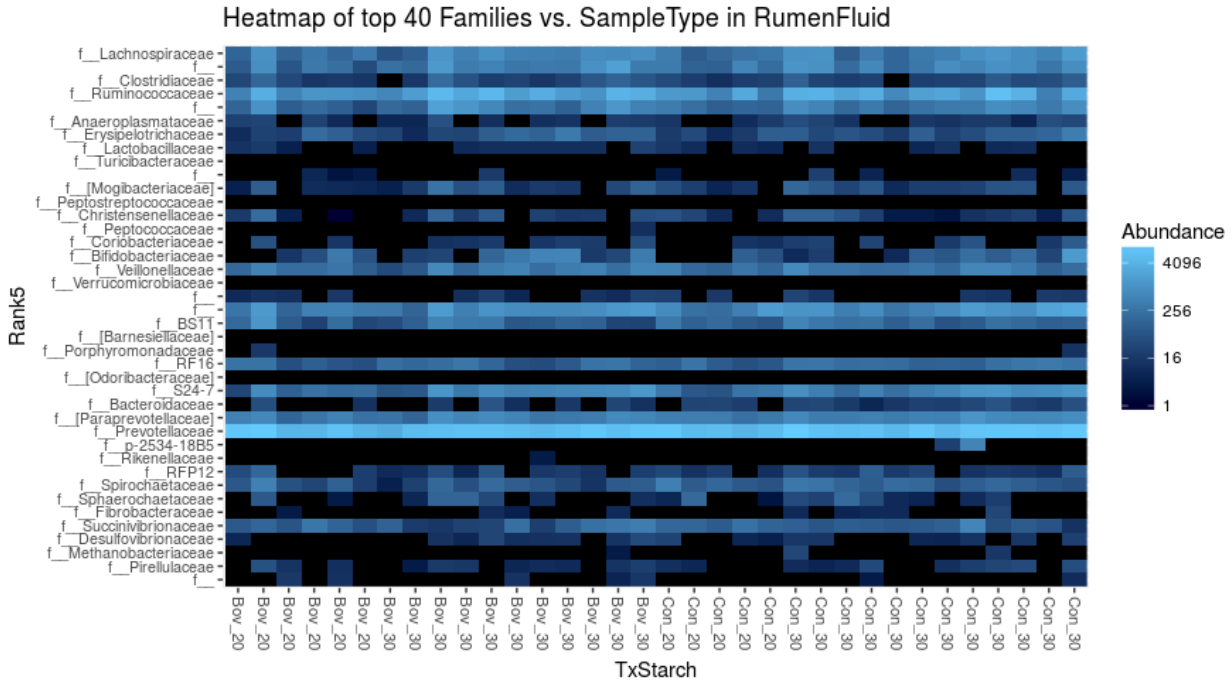


Figure 17. Experiment 2. Multidimensional scaling (MDS) of rumen fluid samples collected during the low starch and the high starch ration by treatment (BOV vs. CON). For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".

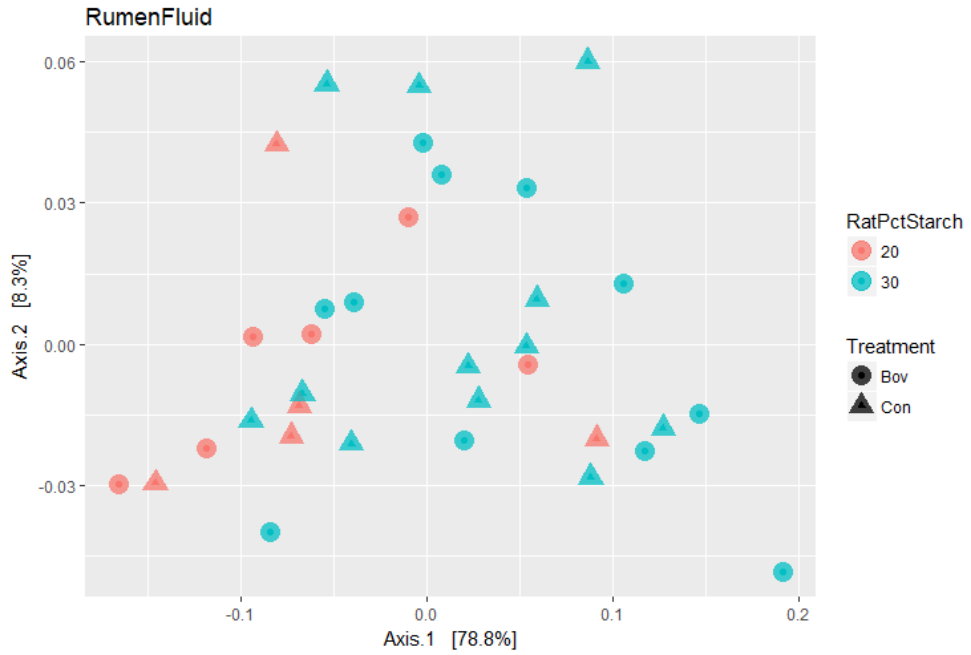


Figure 18. Heatmap of bacteria families identified in fecal samples collected during the low starch ration and the high starch ration by treatment (BOV vs. CON). For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".

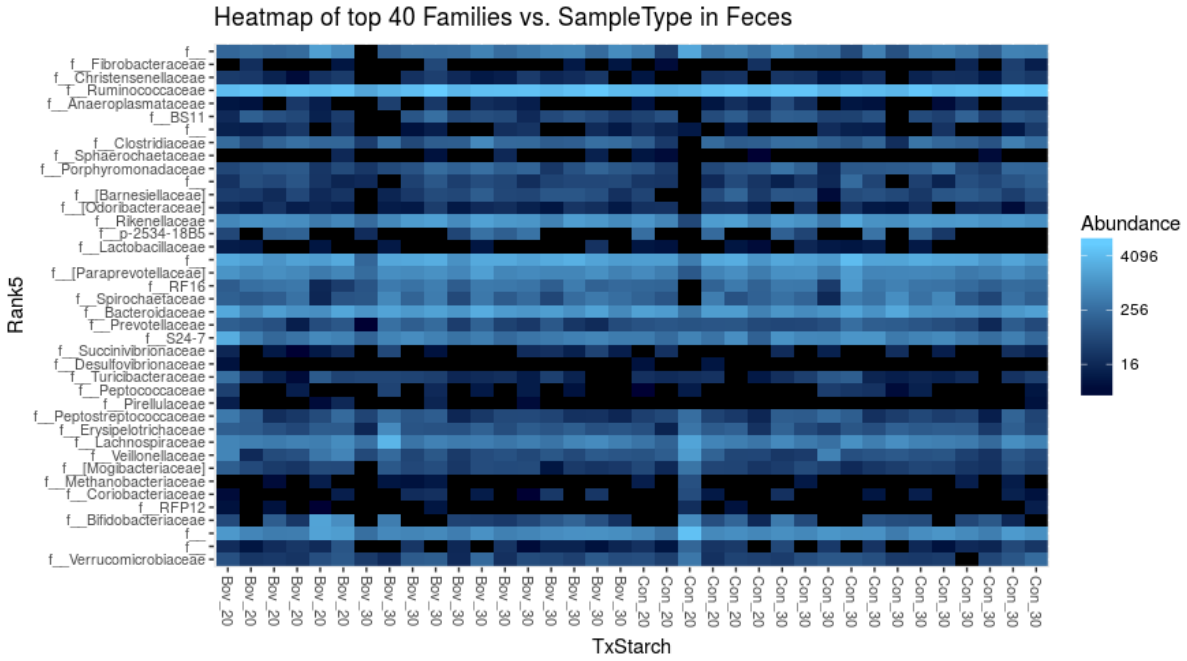
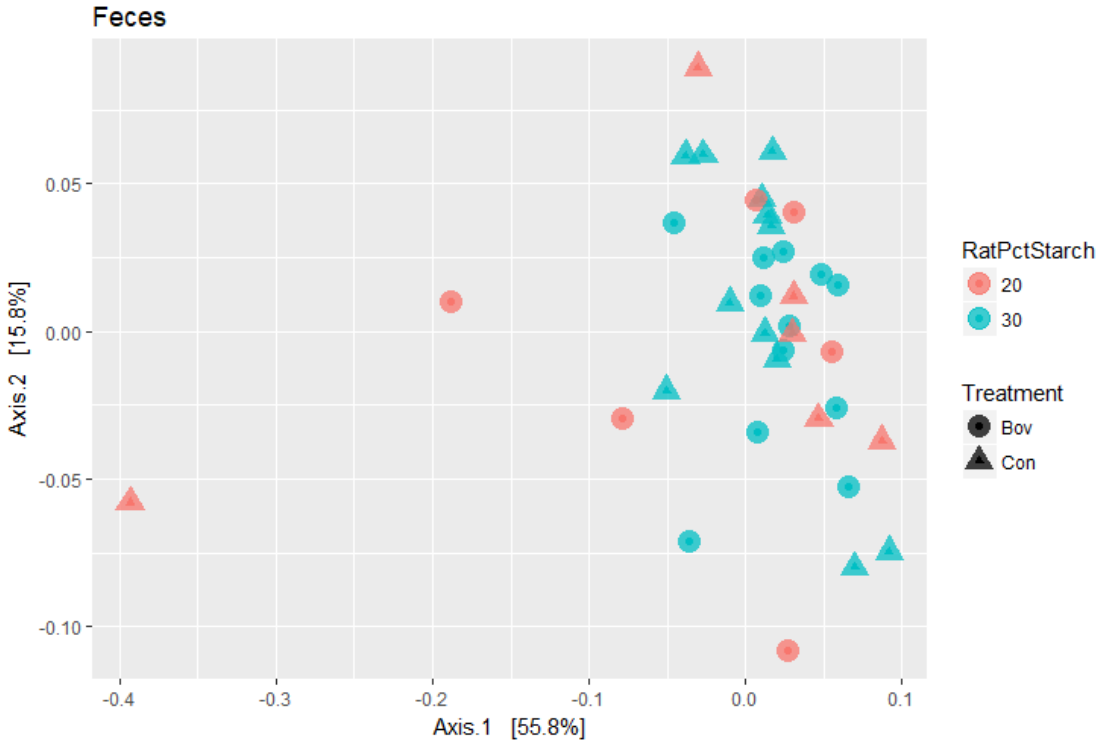


Figure 19. Experiment 2. Multidimensional scaling (MDS) of fecal samples collected during the low and the high starch ration by treatment (BOV vs. CON). For figure simplicity, the low 23.8% starch ration is represented as "20", and the high 31.1% starch ration is represented as "30".



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Appendix

UNIVERSITY OF DELAWARE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

University of Delaware
Institutional Animal Care and Use Committee

Application to Use Animals in Application to use animals in Research

(New and 3-Yr submission)

Title of Protocol: Evaluating the impact of Bovamine on performance, nutrient digestibility, and digestive function in lactating dairy cows	
AUP Number: 66R-2016-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: Tanya Gressley	
Common Name (Strain/Breed if Appropriate): Dairy cows **Genus Species:** Bos taurus	
Date of Submission: 8/24/16	

Official Use Only

IACUC Approval Signature: Tanya Tallon, DVM

Date of Approval: 9/20/16

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. ***(Teaching Protocols, including cooperative extension demonstrations, Exempt)***
12. I understand that by signing, I agree to these assurances. Signature of Principal Investigator — Date: 9/29/16

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

Name	Signature
1. Tanya Gressley	
2. Limin Kung	
3. Stephanie Polukis	
4. Amanda Barnard	
5. MacKenzie Conklin	
6. Richard Morris	
7. Rebecca Savage	
8. Erica Benjamin	
9. Megan Smith	
10. Click here to enter text.	

If after hours participation is required by students on project involving **agricultural animals**, please describe how this is handled and the times and days that students may be on site

Some sampling periods will occur after hours, with specific times outlined in question 4 below. During these times students will work in pairs and will have cell phone numbers for the PIs and farm staff.

The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked "NA" for "Not Applicable."

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

All answers are to be completed using Arial 12 size font.

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:

- Hybridoma/Monoclonal Antibodies ("B")
- Polyclonal Antibodies ("C")
- Survival Surgery ("D")
- Non-Survival Surgery ("E")
- Wildlife Research ("F")

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.

1. Principal Investigator Information:	
a. Name:	Tanya Gressley
b. University/Company:	University of Delaware
c. Department:	Animal and Food Sciences
d. Building/Room:	044 Townsend Hall
e. Office Phone:	302-831-1940
f. Lab Phone(s):	302-831-6214
g. Home Phone:	Click here to enter text.
h. Mobile Phone:	302-750-4289
i. E-Mail Address:	gressley@udel.edu

2. Protocol Status:

a. ☒New Protocol ***OR*** ☐ Re-submission due to three (3) completed years.

If re-submission, enter Protocol Number: Click here to enter text.

b. ☒ Research ***OR*** ☐ Teaching or Cooperative Extension

c. ☐ Laboratory Animals ***OR*** ☐ Wildlife ***OR*** ☒ Agricultural Animals

If "Wildlife" please complete Addendum "F"

For agricultural animal protocols, please list the name and contact information for veterinarian who is on-call . A copy of the protocol should be shared with the veterinarian New Bolton Field Services, 610-925-6310, Primary contact Dr. Billy Smith

d. Proposed Start Date: 9/26/16

e. Proposed Completion Date: 9/25/19

f. Funding Source: To be awarded

g. Award Number if applicable: Click here to enter text.

3. Non-Scientific Summary: In language understandable to a ***high-school senior, very briefly describe*** the goals and significance of this study.

a. Specific Scientific Goals: The mixture of lactic acid producing bacteria (*Lactobacillus acidophilus*) and lactic acid utilizing bacteria (*Propionibacterium freudenreichii*) in the commercially available direct fed microbial, Bovamine, stabilizes the rumen environment and improves performance and feed efficiency in dairy cattle. This proposal outlines two experiments designed to further evaluate performance, feed efficiency, and nutrient digestibility of early lactation dairy cows fed Bovamine (Experiment 1) and to evaluate the ability of Bovamine to stabilize the digestive tract during a ration shift (Experiment 2). For Experiment 1, we hypothesize that inclusion of Bovamine in rations fed to early lactation dairy cattle will increase milk yield, feed efficiency, and nutrient digestibility compared to unsupplemented cows. In addition, we expect the benefit of Bovamine on feed efficiency and nutrient digestibility to increase over time with continued supplementation. In Experiment 2, we hypothesize that Bovamine will stabilize the rumen environment. When switched to a high starch diet, cows fed Bovamine will have reduced changes in rumen pH, rumen VFA, in situ digestibility, fecal starch, and rumen and fecal microbiome compared to unsupplemented cows.

b. Significance of this Research or Teaching/Cooperative Extension Demonstration (including the possible benefits to human and/or animal health, the advancement of scientific knowledge, or the betterment of society): Dairy cows are regularly subjected to ration changes due to changes in animal grouping or feed availability. Direct fed microbials offer the potential to improve nutrient digestibility and stabilize the digestive environment during times of ration change. The results of this experiment will quantify the effects of Bovamine on dairy cow performance, nutrient digestibility, and stability of the gut environment during ration shifts. In Experiment 1, we expect that the Bovamine treatment will increase feed efficiency and total tract nutrient digestibility and reduce fecal starch. In addition, we expect the benefit of Bovamine to increase over time, as these cows will benefit from a stabilized digestive environment compared to the cows on the control treatments. We expect this to be evidenced by greater relative differences between treatments over time in feed efficiency and nutrient digestibility. In Experiment 2, we expect the Bovamine treatment to stabilize changes in the rumen and intestinal environments that will accompany the abrupt change to a higher starch ration. We expect that compared to control cows, those cows fed Bovamine will have reduced changes in rumen pH, rumen VFA, in situ digestibility, fecal starch, and rumen and fecal microbiome. Collectively, results will be directly applicable to dairy cattle feeding programs.

4. Experimental Design: Explain the experimental design. This description should allow the IACUC to understand fully the experimental course of an animal or group of animals from its entry into the experiment to the endpoint of the study.

The inclusion of flow charts, diagrams, and/or tables are greatly encouraged to explain experimental design or sequential events.

Be sure to include all animal events and related details, i.e.,

- **All Procedures**-bleedings, injections, identification methods, genotyping methods, physiological measurements, surgical procedures, euthanasia, etc.
- **Procedural details**–number of animals involved in procedure, approximate animal weight, if relevant (for injections, bleeding, etc.), route, frequency, volume, etc.
- **Pharmaceutical-grade and non-pharmaceutical grade compounds** – Identify any drugs, biologics, or reagents that will be administered to animals.
- **Vaccines and organisms used for challenge** – Identify any experimental or commercial vaccines and/or microorganisms used for challenge of the animals.
- **Federal or other permits** – Identify any federal or other permits needed to obtain vaccines or organisms used in animal studies.
- **Names of surgical procedures** (but reserve the surgical details for the proper Surgical Addenda)

(Describe): Click here to enter text.

Overview: The animal work will be conducted as two separate experiments. Experiment 1 will be conducted to evaluate the effect of Bovamine on performance and nutrient digestibility of high producing, early lactation cows. Experiment 2 will use ruminally cannulated cows to evaluate the impact of Bovamine on nutrient digestibility and digesta microbiome, both during a period of stable ration feeding and following an abrupt ration change.

Experiment 1 (30 cows, Table 1):

Animals and treatments. Early lactation (40-160 days in milk) Holstein dairy cows (n = 30, approximately 20 multiparous and 10 primiparous) will be housed in a 30-cow deep sand bedded freestall barn and fed using individual Calan gates. Cows will be fed once daily (~0800 h) for ad-libitum intake and refusals will be removed and weighed daily for measurement of daily intake. Cows will be milked twice daily (~0430 and 1600 h) with milk weights recorded at each milking. Cows will be weighed monthly on two consecutive days.

The experiment will be conducted over 12 weeks, with a 2 week baseline period followed by a 10 week experimental period. During the baseline period, all cows will be fed a total mixed ration without direct fed microbials (DFM). The ration will be formulated by Ian Shivas at Renaissance Nutrition in collaboration with Drs. Kung and Gressley. At the end of the baseline period, cows will be blocked by parity and days in milk and randomly assigned to one of two treatments according to a randomized block design. 15 cows will be assigned to the control treatment and 15 will be assigned to the Bovamine treatment. During the treatment period, cows on the control treatment will continue to be fed the ration without DFM while cows on the Bovamine treatment will receive the same ration but supplemented with Bovamine ($3x10^9$ CFU/head/d). Cows will remain on their respective ration until the completion of the experiment. Cows on the Bovamine treatment will be supplemented with Bovamine as a twice daily topdress given ~8 am and 5 pm. Bovamine will be mixed with a carrier approximately 100 g of corn grain prior to being topdressed onto the ration. At 8 am the Bovamine will be placed over the freshly delivered ration and at 5 pm the Bovamine will be placed over existing uneaten ration from the morning feeding. Cows on the control treatment will receive carrier only. Topdress treatments will be prepared once weekly in the lab by weighing the Bovamine and carrier (Bovamine treatment) or carrier only (control treatment) into individual bags labeled with each cow number (14 bags per cow per week, with one bag for each cow for each am and pm feeding).

Milk sampling. Milk samples (720 total) will be collected at both milkings on a consistent day each week and mailed to Dairy One for NIR analysis of fat, true protein, lactose, MUN, and SCC.

Digestibility and fecal starch measures. A subset of 14 cows (7 per treatment) will be used for measurement of total tract apparent nutrient digestibility at the end of the baseline period (wk -1), at 1 and 2 weeks following the start of the treatment period (wk 1, 2), and every 2 weeks thereafter (wk 4, 6, 8, 10). On the last day of each of those weeks, fecal grab samples will be collected at 0900, 1500, 2100, and 0300 h. Artificial insemination gloves lubricated with a water-based breeding lubricant will be used and samples will be collected via rectal palpation. Fecal samples will be frozen, composited into 1 sample per cow per day, and analyzed to determine fecal starch content and nutrient digestibility. For the remaining 16 cows, fecal samples will be collected on the same dates but at only one time point (0900 h) and used for evaluation of fecal starch. At that same time (0900 h), fecal score (1=liquid to 5=extremely well formed) will be evaluated on all 30 cows.

Table 1. Data collection and sampling during Experiment 1.

	Week during baseline period (30 cows, no DFM)		Week during treatment period (15 cows fed no DFM, 15 cows fed Bovamine)									
	-2	-1	1	2	3	4	5	6	7	8	9	10
Daily intake	X	X	X	X	X	X	X	X	X	X	X	X
Daily milk yield	X	X	X	X	X	X	X	X	X	X	X	X
Weekly milk samples	X	X	X	X	X	X	X	X	X	X	X	X
Fecal score and fecal starch		X	X	X		X		X		X		X
Digestibility measurement (14 cows only)		X	X	X		X		X		X		X

Experiment 2 (6 cows; Table 2)

Animals and treatments. Experiment 2 will use ruminally cannulated mid lactation (~200 DIM) Holstein dairy cows (n = 6) housed in tie stalls. Attempts will be made to use cows with pre-existing rumen cannulas. It is expected that 3 cows with existing rumen cannulas will be available and an additional 3 cows will need to be ruminally cannulated prior to the start of this experiment.

Cows will be fed twice daily (~0900 and 1630 h) for ad-libitum intake and refusals will be removed and weighed daily for measurement of daily intake. Cows will be milked twice daily (~0430 and 1600 h) with milk weights recorded at each milking.

The experiment will be conducted as a crossover design with two 6-week periods. During each 6-week period cows will be assigned to either Bovamine plus carrier or carrier only as described in Experiment 1. During the first period, 3 cows will be on the control treatment and 3 cows will be on the Bovamine treatment, and treatments will be switched for the second period. The first 5 weeks of each period will be used for adaptation to Bovamine and the last week will be used to simulate an abrupt rations shift. During the first 5 weeks of each period cows will be fed a ration containing 22% starch, then cows will be abruptly switched to a ration containing 26% starch for the 6th week. Rations will be formulated with the assistance of Ian Shivas as described above. 22% starch is a relatively normal level of starch feeding whereas 26% starch is on the upper end of an acceptable level

of starch. The 26% starch ration is expected to cause a depression in rumen pH and may result in subacute rumen acidosis as defined as a rumen pH less than 5.6 for greater than 180 minutes per day. However the 26% starch ration is not expected to cause any short-term or long-term negative consequences on animal health nor is it expected to cause any clinical symptoms.

Milk sampling. During each period milk samples (192 total) will be collected at both milkings on a consistent day each week during weeks 1-5 and on days 1, 3, and 7 of week 6 and mailed to Dairy One for analysis.

Rumen and fecal measures. Rumen pH will be continuously measured during weeks 5 and 6 in all 6 cows using indwelling pH meters (DASCOR, Inc.). These weighted boluses sit at the base of the rumen and will be attached to the rumen cannula plug with a string for ease of recovery. Boluses will be removed three times a week for data downloading, to check calibration, and to re-calibrate if necessary.

In situ rumen digestibility, rumen volatile fatty acids (VFA), fecal pH, fecal starch, and rumen and fecal microbiome will be determined during weeks 5 and 6. In situ digestibility will be measured at the end of week 5, beginning of week 6, and end of week 6. Dried and ground samples of the 22% starch ration will be placed in Dacron bags in the rumen and incubated for 6, 12, 18, and 24 h for measurement of dry matter disappearance. A lingerie bag weighted down with rocks will be used to contain the bags, and triplicate bags containing 10 grams of dried ration will be evaluated at each time point. Timing of bag placement will occur such that timing of placement in the rumen is staggered but all bags will be removed from the rumen at the same time.

At the time of bag placement rumen and fecal samples will be collected for measurement of rumen VFA (144 total samples), fecal pH (144 total measurements), and fecal starch (composited by cow and sampling day, 36 total samples). Rumen fluid samples will be collected using a gloved arm and approximately 300 mL of rumen fluid will be removed at each time point. Fecal samples will be collected by rectal palpation as described for Experiment 1. Subsamples of rumen fluid and feces collected at one time point on each of those days will be saved for microbiome analysis.

Table 2. Ration starch concentration, data collection and sampling during Experiment 2.

	Week during period 1 (6 cows, 3 fed no DFM, 3 fed Bovamine)						Week during period 2 (6 cows, opposite treatment from period 1)					
	1	2	3	4	5	6	1	2	3	4	5	6
Ration starch %	22	22	22	22	22	26	22	22	22	22	22	26
Daily intake	X	X	X	X	X	X	X	X	X	X	X	X
Daily milk yield	X	X	X	X	X	X	X	X	X	X	X	X
Milk samples	d 7	d 7	d 7	d 7	d 7	d 1, 3, 7	d 7	d 7	d 7	d 7	d 7	d 1, 3, 7
Continuous rumen pH measurement (4 cows only)					X	X					X	X
In situ digestibility, rumen VFA, fecal pH, fecal VFA, and fecal microbiome					d 7	d 1, 7					d 7	d 1, 7

5. Administration of compounds

Drug name or class of drug	Volume	Dose or range of doses	Route (IP, IV, SC, IM, PO)	Frequency	Duration	Pharma-grade Yes or No

If non-pharmaceutical grade compounds are used, they must be justified (such as pharmaceutical-grade not available) and the method to ensure appropriate preparation must be described: (for example: pharmaceutical grade drugs are not available. Sterile saline will be used as a vehicle and the solution will be sterile-filtered. Click here to enter text.

6. Does this work involve surgery or antibody production ☒ Yes ☐ No

If yes, please complete Addendum B for hybridoma/monoclonal antibody production, Addendum C for polyclonal antibody production, Addendum D for survival surgery and Addendum E for terminal surgery

REFINEMENT, REDUCTION & REPLACEMENT

When using animals for research, it is important to consider the three Rs: reduction, refinement, and replacement to reduce both animal distress and the number of animals used in the laboratory.

Reduction: Minimizing the number of animals used
Refinement: Using techniques and procedures to reduce pain and distress
Replacement: Using non-animal methods or lower phylogenetic organisms

7. Justification for the Use of Animals (instead of *in vitro* methods)
(Check all that apply and explain):

a. ☒ The complexity of the processes being studied cannot be duplicated or modeled in simpler systems: ***(Explain)***: The experiments outlined above are designed to evaluate the effectiveness of a direct fed microbial on dairy cattle performance and nutrient digestibility. Model systems do not exist to evaluate the impact of feed supplements on digestive physiology in dairy cattle.

b. ☐ There is not enough information known about the processes being studied to design non-living models: ***(Explain)***: Click here to enter text.

c. ☐ Other: ***(Explain)***: Click here to enter text.

8. Justification for Species Appropriateness:
(Check all that apply and explain):

a. ☐ A large database exists, allowing comparisons with previous data: ***(Explain)***: Click here to enter text.

b. ☒ The anatomy or physiology is uniquely suited to the study proposed: ***(Explain)***: Bovamine is a direct fed microbial specifically formulated to enhance feed digestion in dairy and beef cattle. It would be impossible to evaluate the efficacy of a product designed for cattle using a different species as a model.

c. ☐ This is the lowest species on the phylogenic scale suitable to the proposed study: ***(Explain)***: Click here to enter text.

d. ☐ Other: ***(Explain)***: Click here to enter text.

9. Justification for Number of Animals Requested: (Note: numbers should include animals used for breeding and all animals born)

a. ☒ Pilot study or preliminary project where group variances are unknown at the present time. Describe the information used to estimate how many animals will be needed: (Only a limited number of animals will be permitted.)
(Explain): Experiment 2 has been developed as a pilot study. There are currently no studies that exist that have evaluated the impact of Bovamine on rumen and fecal microbiome. While we expect to find differences in microbiome of supplemented cows, the magnitude of these differences is unknown and results of the current study will be used to design future studies. We did, however, conduct a power analysis on other rumen and fecal measures to be evaluated. Based on typical standard errors we observe, we expect to be able to detect differences of 10 mM in total VFA, 0.20 in rumen pH, and 0.25 in fecal pH. While these differences are fairly large in magnitude, we expect treatment differences may approach these values during the transition to the 26% starch ration.

b. ☒ Group sizes are determined statistically. Describe the statistical analysis used to estimate the number (N) of animals needed: N may be estimated from a power analysis for the most important measurement in the study, usually based on the expected size of the treatment effect, the standard error associated with the measurement, and the desired statistical power (e.g. $P < 0.05$). Data analysis methods should not be submitted unless directly applicable to the estimate of N.

An online calculator may be found at: http://www.math.uiowa.edu/~rlenth/Power/
or a stand-alone calculator that can be downloaded from
http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3

(Explain): Group sizes in Experiment 1 were determined using a power analysis. For the full experiment with 30 cows, Bovamine is expected to increase milk yield by approximately 4%, or 2 kg/d. Assuming an expected standard deviation of 2 kg/d in this experimental structure, alpha of 0.05 and a power of 0.80, 15 cows per treatment should be adequate to detect this difference. A power analysis was also used to determine the subset of 14 cows (7 per treatment) to be used for digestibility measures. Specifically, we expect dry matter digestibility to increase with Bovamine feeding by 2.8 percentage units. Assuming a standard deviation of 2 percentage units and the same alpha and power as above, 7 cows per treatment should be sufficient to detect that difference.

c. ☐ Group sizes are based on the quantity of harvested cells or the amount of tissue required for *in vitro* studies. Explain how much tissue is needed based on the number of experiments to be conducted and the amount of tissue you expect to obtain from each animal (e.g., 10g of tissues are needed: Each animal can provide 2g. 10g /2g per animal = 5 animals needed.) ***(Explain)***: Click here to enter text.

d. ☐ Teaching or cooperative extension demonstration protocol. Specify the number of students in the class, the student to animal ratio and how that ratio was determined: Animal numbers should be minimized to the fullest extent possible without compromising the quality of the hands-on teaching experience for students or the health and welfare of the animals. ***(Explain)***: Click here to enter text.

e. ☐ Study involving feral or wild animals. Animals will be captured and released in an attempt to maximize the sample size within logistical constraints. Describe the process by which you estimate these numbers and estimate the precision needed: ***(Explain)***: Click here to enter text.

f. ☐ Observational, non-manipulative study. Animals will not be captured, their behavior will not be interfered with, and exact animal numbers cannot be predicted: ***(Explain)***: Click here to enter text.

g. ☐ Product testing. The number of animals needed is based on FDA or USDA guidelines. Provide the citation from the regulations, the IND tracking number, or relevant FDA or USDA correspondence: ***(Explain)***: Click here to enter text.

h. ☐ Other. Elaborate, indicating the method used to determine the group size. ***(Explain)***: Click here to enter text.

10. Animals Requested:

Common Name	Genus and Species	Total Number of Animals for Three Years
1. Dairy cows	Bos taurus	36
2. Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.

11. Where will animals be obtained and are there any special shipping requirements? from the UD dairy farm

If these are privately owned animals please attach an owner consent form

Are agricultural animals obtained from a non-traditional source such as poultry from a commercial production company or swine from commercial herd? ☐ Yes ☒ No

If **yes**, please describe how the animals are tested and determined to be free of diseases which could potentially infect other animals on site, and any special precautions, such as quarantine isolation housing that is required. Click here to enter text.

12. Where will animals be housed (or captured for wildlife)? Experiment 1 cows will be housed in the Calan barn on the dairy and Experiment 2 cows will be housed in the tiestalls on the dairy.

13. Will any untreated or non-manipulated animals be humanely euthanized, to obtain tissue, cells, etc.? ☐ Yes ☒ No

If **Yes,** list types of tissue, etc: Click here to enter text.

14. Dietary Manipulations ☒ Yes ☐ No

If **Yes,** list and explain (Note: if food or fluid will be restricted, describe method for assessing the health and wellbeing of the animals. Body weights must be recorded at least weekly. Amount earned (if animals work for food or fluid) during testing and amount freely given must be recorded. A scientific justification must be provided for departures from the recommendations of the Guide.) Cows will either be fed a control ration or a ration supplemented with a direct fed microbial as described above. In experiment 2, cows will be abruptly switched from a 22% starch ration to a 26% starch ration.

15. Environmental Stress (e.g. cold, prolonged restraint, forced exercise, shock) ☐ Yes ☒ No

If **Yes,** list and explain: Click here to enter text.

16. Special Study Requirements or Exceptions to Standards: Please describe any special study requirements such as single housing of the animals, exemption from environmental enrichment, or special caging None

17. Will any animal undergo anesthesia for any reason other than surgery? ☐ Yes ☐ **XXX**No

If **Yes,**

a. List Procedures and Reason(s) for using anesthesia: Click here to enter text.

b. Check the type of anesthesia to be used.

☐ Isoflurane

☐ Injectable ***(For injectable, complete the following):***

Drug: Click here to enter text.
Dose: Click here to enter text.
Route: Click here to enter text.

HAZARDOUS AGENTS

18. Administration of Hazardous Chemicals, Drugs, Toxins, or Nanoparticles

☐ Yes CAS#_____ ☒ No

If **Yes,** describe hazards posed to personnel: Click here to enter text.
Methods to control exposure: Click here to enter text.
Methods of Disposal of Animals and Bedding: Click here to enter text.

19. Administration of radioactive materials ☐ Yes ☒ No

a. Type to be used. Include radioisotope(s) and chemical form(s): Click here to enter text.

b. Describe the practices and procedures to be followed for minimization of radiation exposure to workers and for the handling and disposal of contaminated materials associated with this study:

(Include the methods for management of radioactive wastes and monitoring facility for radioactive contamination, if applicable.) Click here to enter text.

c. Who will be responsible for the daily care of animals containing radioactive materials?

Click here to enter text.

d. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Click here to enter text.

Please attach a copy of any approvals or provide the approval number.

Click here to enter text.

20. Study of Irradiation *in vivo*? ☐ Yes (gamma irradiator? ☐ or x-ray irradiator? ☐)

☒No

a. Make, model, and location of irradiator to be used:

Click here to enter text.

b. Approval received from UD- Environmental Health and Safety? ☐ Yes ☐ No ☐ Pending

Please attach a copy of any approvals or provide the approval number.Click here to enter text.

21. Administration of Biological Agents (eg microorganisms, recombinant DNA, **HUMAN** serum, tissue, cell lines, etc.) ☐ Yes ☒No

Animal Biosafety Level ☐ 1 ☐ 2 ☐ 3 ☐ 4

Source of Biological Agents: Click here to enter text.

Describe hazards posed to personnel:Click here to enter text.
Methods to control exposure:Click here to enter text.
Methods of Disposal of Animals and Bedding:Click here to enter text.
Approval received from UD- Institutional Biosafety Committee, and if required, the UD-Select Agent Committee? ☐ Yes ☐ No ☐ Pending
Please attach a copy of any approvals or provide the approval number. Click here to enter text.

22. Will tumor cells, tissue, sera, viral vectors or other biologics of RODENT origin – other than those isolated from rodents already housed in the facility – be administered to animals?

☐ Yes ☒ No

If Yes, this material must be tested for rodent pathogens and test results must be attached (Please contact the Attending Veterinarian for details).

23. Use of Genetically Engineered (GEM, transgenic, knockout) Animals

☐ Yes ☒ No

If Yes, please describe any anticipated phenotypes that may cause pain or distress and any special care or monitoring that the animals will require.

Click here to enter text.

Does the proposed work involve creating new genetically modified animals, or involve crossing two genetically modified animals to produce offspring with a new genotype.

☐ Yes ☐ No

Approval received from UD- Institutional Biosafety Committee?

☐ Yes ☐ No ☐ Pending ☐ Exempt (breeding of two lines of genetically-modified rodents is exempt if 1) both parents can be housed under BL1 containment and 2) neither parent strain incorporates more than one half of the genome of an exogenous eukaryotic virus or incorporates a transgene under the control of a gammaretroviral long terminal repeat and 3)the rodent that results from the breeding is not expected to contain more than one half of an exogenous viral genome)

Please attach a copy of any approvals or provide the approval number.

Click here to enter text.

Potential Pain and Distress

24. Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: *(Note change of categories from previous form)*	
Category	**Description**
☐ **B**	Breeding or holding where NO research is conducted
☐ **C**	Procedure involving momentary or no pain or distress
☒ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

25. If animals may experience pain or distress, (for example, animal challenge studies using a pathogenic disease agent) please include how they will be monitored, frequency of observation, and potential treatments (note: for survival surgery procedures this will be described in addendum D and does not need to be repeated here) See Addendum D

26. Please describe criteria for when an animal will be euthanized (humane endpoints – possible examples include 20% weight loss, ulceration of subcutaneous tumors, difficulty ambulating, hunched posture);

Euthanasia is not expected to be required as a result of this work. Should a cow appear to be in distress (for example greater than 20% reduction in feed intake or observed physical ailment), dairy manager Richard Morris will be informed. He will monitor or treat the cow or call New Bolton Field Service as necessary.

Alternatives to Pain and Distress

27. If you have indicated that **animals in your study experience pain or distress (category D or E),** even if it will be fully alleviated, please mark the appropriate check boxes below and fill in the requested information for each item marked. (Note: If the pain category is B or C, please skip to question 28)

You must conduct at least two (2) searches.

I have considered alternatives to the use of animals in my study. Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate. I have used the following methods and sources to search for alternatives:

Note: You may need to do more than one search per database to look for alternatives if there are multiple procedures that may cause pain and/or distress.

Database Used:

☒ **Medline** ☐ **Agricola**

☐ **Toxline** ☐ **CAB Abstracts**

☐ **Biosis** ☐ **Other *(Specify)***: Click here to enter text.

Date of Search: 8/24/16

Years Covered: all

Keywords Used (must include the word *alternative*): rumen cannulation and alternative

Number of Papers Found: 3

Discussion of the Relevancy of the Papers Found: One paper described a non-invasive system to monitor ruminal temperature. The other two actually used animals with rumen cannulas and were flagged for the use of the word alternative for other reasons (one was about urinary catherization in sheep and the other was about endogenous phosphorus loss).

Database Used:

☐ **Medline** ☐ **Agricola**

☐ **Toxline** ☐ **CAB Abstracts**

☐ **Biosis** ☒ **Other *(Specify)***: Web of Science

Date of Search: 8/24/16

Years Covered: all

Keywords Used (must include the word *alternative*): rumen and (fistula or cannula) and alternative

Number of Papers Found: 25

Discussion of the Relevancy of the Papers Found: The majority of the papers found (18) were using cannulated ruminants to evaluate some aspect of feeding. Two papers pertained to in vitro models (where rumen fluid was taken from animals and incubated in the lab) and three papers were completely unrelated to ruminant nutrition. Two papers were relevant. One paper (Anim Feed Sci Tech 2014 198:57-66) evaluated the use of stomach tubing to collect rumen contents and avoid rumen cannulation. They used sheep and goats with rumen cannulas and analyzed rumen fluid collected both via stomach tube and directly from the rumen. The results suggest that stomach tubing provided similar results but results should be interpreted with caution. It is the experience of the PIs involved in this protocol that samples collected by stomach tube and are not currently accepted by the research community due to their contamination with saliva. The other relevant paper was a French bulletin entitled "Interest and limitations of techniques replacing experimental surgery on the digestive tract of herbivores". I only had access to the abstract and the abstract concludes that alternative approaches can only partially replace the use of digestive surgery.

Unnecessary Duplication of Work.

28. Activities involving animals must not unnecessarily duplicate previous experiments performed by you or others. Provide a written narrative that assures that the activities of this project comply with this requirement and support this assurance by performing a literature search.

The search should return, at minimum, the related previous work from your laboratory.

You must conduct at least two (2) searches.

(NOT REQUIRED FOR TEACHING PROTOCOLS)

Note: You may need to do more than one search per database to look for duplication of work, especially if you are doing more than one experiment.

Database Used:

☒ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☐ **Other *(Specify)***: Click here to enter text.

Date of Search: 8/24/16

Years Covered: all

Keywords Used: acidophilus and freudenreichii and cow

Number of Papers Found: 13

Discussion of the Relevancy of the Papers Found: As explanation for the search terms, Bovamine contains two direct fed microbial species, *Lactobacillus acidophilus* and *Propionibacterium freudenreichii*. Ten of the papers were related to using these direct fed microbials to reduce E coli or Salmonella shedding by beef cattle. One paper was related to performance of beef cattle fed these direct fed microbials. Two papers were relevant to dairy cattle nutrition. One paper (J Dairy Sci 2011 94:4616-4622) found that there was a benefit of feeding direct fed microbials on nutrient digestibility in cows undergoing heat stress. The other (J Dairy Sci 2007 90:1802-1809) had objectives that were similar to our Experiment 1 except that they used cows in midlactation.

Database Used:

☒ **Medline**	☐ **Agricola**
☐ **Toxline**	☐ **CAB Abstracts**
☐ **Biosis**	☒ **Other *(Specify)***: Web pf Science

Date of Search: 8/24/16

Years Covered: all

Keywords Used: acidophilus and freudenreichii and cow

Number of Papers Found: 8

Discussion of the Relevancy of the Papers Found: 4 of the results were actually of the same relevant paper described above (J Dairy Sci 2007 90:1802-1809). Of the other 4 results, 1 was the other relevant paper described above, 1 was related to culturing those organisms, and 2 were related to the impact of those organisms on fatty acid metabolism in the rumen.

Disposition of Animals

29. What is the expected disposition of animals at the end of the experiments? ***(Check all that apply)***:

☐ Euthanized - If an infectious disease studies - carcasses decontaminated by ☐ incineration
☐ composting ☐ other Click here to enter text.

☒Maintained

☐ Released ***(Wildlife Only)***

☐ Other ***(Specify)***: Click here to enter text.

30. Euthanasia*

Select methods that will be used in case of emergency and/or at the end of the procedure/experiment.

***NOTE:**

- Methods must be approved by the AVMA or must be scientifically justified.
- A "Primary" and "Secondary" method must be selected (UD Double Kill Policy).
- **If different methods will be used for different groups** of animals, indicate the group after the procedure (e.g., write "Neonates" after Decapitation, "Adults" after CO_2, "Terminal Surgery Animals" after Isoflurane Anesthesia Overdose, etc.).

☐ Animals will NOT be under anesthesia when euthanasia is performed.

☐ Animals will be under anesthesia when euthanasia is performed. ***(Check drug used below)***:

☐ Isoflurane

☐ Injectable ***(Complete the following)***:

Drug: Click here to enter text.
Dose: Click here to enter text.
Route: Click here to enter text.

PRIMARY method(s) of euthanasia

☐ CO_2 by compressed gas cylinder ***(Not for animals already under anesthesia or neonates)***

☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg ***(Check route below)***:

☐ IV ☐ IP ☐ IC

☐ Isoflurane Anesthesia Overdose - Inhalant

☐ Cervical Dislocation ***(acceptable with anesthesia, or for poultry, without anesthesia if personnel are trained)***

☐ Decapitation ***(only under anesthesia or neonates)***

☐ Exsanguination or Perfusion ***(only under anesthesia)***

☐ Incision of Chest Cavity – Bilateral Pneumothorax ***(only under anesthesia)***

☐ Pithing – ***(only under anesthesia) (amphibians, reptiles only)***

☐ Removal of Vital Organ(s) ***(only under anesthesia) (Check all that apply):***

☐ Brain ☐ Kidneys

☐ Heart ☐ GI Tract

☐ Liver ☐ Lungs

☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.

☒Other Method of Euthanasia: ***(Describe and Scientifically Justify)***: Euthanasia will never be conducted by anybody involved in this experiment. Should euthanasia be required, it will be conducted by ~~dairy manager Richard Morris or~~ New Bolton Field Service by administration of euthanasia solution.

SECONDARY method(s) of euthanasia that will be used to ensure that the animal does not survive:

☐ Cervical Dislocation

☐ Decapitation

☐ Exsanguination or Perfusion

☐ Incision of Chest Cavity – Bilateral Pneumothorax

☐ Barbiturate Euthanasia Solution - Injectable ≥150mg/kg **(*Check route below)***:

☐ IV ☐ IP ☐ IC

☐ Pithing – Double pithing required ***(fish, amphibians, reptiles only)***

☐ Monitor for lack of respiration and heart beat (Agricultural animals only)

☐ Removal of Vital Organ(s): ***(Check all that apply)***:

☐ Brain ☐ Kidneys

☐ Heart ☐ GI Tract

☐ Liver ☐ Lungs

☐ Other Vital Organ(s) – ***(Specify):*** Click here to enter text.

☐ Other Method of Euthanasia: ***(Describe and Scientifically Justify)***: Click here to enter text.

Personnel and Training

31. Personnel involved in Protocol *(Include Principal Investigator):*

***Status*:** Indicate Prof, Post-Doc, Grad Student, Lab Manager, Research Assistant, Technician, etc.

***Qualifications*:** Include **procedures this person is proficient in performing** on proposed species and the time they have been doing the procedure.
Be specific (e.g. sub-mandibular bleeding on mice-2yrs, performing castrations on mice and rats-1yr, tail-vein injections on mice-2yrs, etc.) **(If no experience, list who will train.)**

***Responsibilities*:** Include **all responsibilities** this person will have with live animals on this protocol, including euthanizing animals.

Name	**E-mail**	**Office phone number**	**Home/Cell phone number**	**Received IACUC-required training**
Tanya Gressley	gressley@udel.edu	302-831-1940	302-750-4289	**Yes ☒ No ☐**

Status: Associate professor

Qualifications: Over 15 years experience in conducting nutrition studies including working with ruminally cannulated cows.

Responsibilities: Will oversee all animal work and assist when needed.

Name	E-mail	Office phone number	Home/Cell phone number	Received IACUC-required training
Limin Kung, Jr	lksilage@udel.edu	320-831-2524	302-388-5589	Yes ☒ No ☐

Status: Professor

Qualifications: Over 30 years of experience working on nutrition studies.

Responsibilities: Will assist with trial management and trial decisions as needed.

Name	E-mail	Office phone number	Home/Cell phone number	Received IACUC-required training
Stephanie Polukis	spolukis@gmail.com	302-831-2269	302-584-6147	Yes ☒ No ☐

Status: 2nd year PhD Student

Qualifications: Three years of experience working with nutrition studies and ruminally cannulated cows.

Responsibilities: Will be the primary student in charge of Experiment 1. Will train the other students listed on this protocol regarding the specific animal care procedures for Experiment 1.

Name	E-mail	Office phone number	Home/Cell phone number	Received IACUC-required training
Amanda Barnard	Pocomoke@udel.edu	302-831-6214	302-668-7940	Yes ☒ No ☐

Status: 2nd year PhD student

Qualifications: 4 years of experience working with nutrition studies and with ruminally cannulated cows.

Responsibilities: Will train MacKenzie Conklin on all animal care aspects of this protocol. Will assist with training the other students listed on this protocol regarding the specific animal care procedures for Experiment 2.

Name	E-mail	Office phone number	Home/Cell phone number	Received IACUC-required training
MacKenzie Conklin	mkconkli@udel.edu	302-831-6214	845-494-1378	Yes ☐ No ☒

Status: Incoming MS student

Qualifications: 2 years of experience at North Carolina State University working at their dairy teaching and research unit. Duties included assisting with research trials and regular animal care.

Responsibilities: Will be the primary student in charge of Experiment 2. Will be trained by Amanda Barnard and Tanya Gressley. Will assist with training the other students listed on this protocol regarding the specific animal care procedures for Experiment 2.

Name	**E-mail**	**Office phone number**	**Home/Cell phone number**	**Received IACUC-required training**
Richard Morris	rmorris@udel.edu	302-831-2510	302-218-3039	**Yes ☒ No ☐**

Status: Research Associate III, Dairy farm manager

Qualifications: Over 30 years of experience working with and managing dairy cattle. Over 25 years of experience assisting with nutrition experiments at the University of Delaware.

Responsibilities: Oversees that day to day care of all animals on the dairy and makes management decisions as necessary. Feeds or coordinates feeding of cows on the research experiment. Responsible for post-operative monitoring and care of cows following cannulation surgery.

Name	**E-mail**	**Office phone number**	**Home/Cell phone number**	**Received IACUC-required training**
Rebecca Savage Erica Benjamin Megan Smith	bsavage@udel.edu ericab.bio@gmail.com mlsmith@udel.edu	302-831-2269 (all 3)	302-898-7770 302-407-1147 **609-577-3520**	**Yes ☒ No ☐**

Status: 3rd year MS student; 3rd year PhD student; 4th year PhD student

Qualifications: Rebecca has over 3 years of experience working with nutrition studies and with ruminally cannulated cows. Erica has 6 months of experience working with nutrition studies and with ruminally cannulated cows. Megan has over 6 years of experience working with nutrition studies and with ruminally cannulated cows.

Responsibilities: All students will assist with animal care, treatment administration, and sampling.

University of Delaware
Institutional Animal Care and Use Committee

Application to Use Animals in Research and Teaching

ADDENDUM "D"

Survival Surgery

(*Please use a separate form for each surgical procedure and each species.*)

AUP Number: 66R-2016-0	**← (4 digits only — if new, leave blank)**
Project: Evaluating the impact of Bovamine on performance, nutrient digestibility, and digestive function in lactating dairy cows	

General Information

1. Name of survival surgical procedure: rumen cannulation

2. Reason for performing this procedure: to provide an access to the rumen to allow for rumen fluid sampling and rumen pH measurement

3. Species: **Bos taurus**

4. Total maximum number of animal undergoing this surgical procedure over 3 years:
3

5. Location of the surgery:
a. Building: UD dairy farm
b. Room number: NA

6. Type of Surgery: *(choose one)*

☐ Minor Operative Surgery

☒ Major Operative Surgery
(Opening a body cavity, opening the cranium, or producing substantial impairment)

7. Will any animals undergo more than one MINOR survival surgery?

☐ Yes ☒No

If Yes. complete the following:

Maximum number of surgeries an animal will undergo:	Click here to enter text.
Type(s) of surgeries that the animal will undergo:	Click here to enter text.
Time interval between surgeries:	Click here to enter text.
Justify need for multiple surgeries: Click here to enter text.	

8. Will any animals undergo more than one MAJOR survival surgery?
(Strongly Discouraged)

☐ Yes ☒No

If Yes. complete the following:

Maximum number of surgeries an animal will undergo:	Click here to enter text.
Type(s) of surgeries that the animal will undergo:	Click here to enter text.
Time interval between surgeries:	Click here to enter text.
Scientific justification for the need for multiple major surgeries: Click here to enter text.	

Medication and Fluid Administration
(not anesthetics and analgesics)

9. Will neuromuscular blocking agent(s) be used?

☐ Yes ☒No

If **Yes**, complete the following

Agent(s):	Click here to enter text.

Dose: (mg/kg)	Click here to enter text.
Route of Administration:	Click here to enter text.
Approximate length of time animal will be under the influence of the agent:	Click here to enter text.

Description of how/when agent will be administered:

Click here to enter text.

Description of mechanical ventilation while neuromuscular blocking agent is in effect (include equipment, tidal volume and respiration rate):

Click here to enter text.

Scientific Justification for use of the agent:

Click here to enter text.

10. Will any drugs or agents (OTHER THAN anesthetics or analgesics) be administered during surgery (e.g. antibiotics, atropine, saline, specific drugs or agents as part of the experiment)?

☐ Yes ☒ No

If **Yes**, complete the following for each drug:

Drug:	Click here to enter text.
Dose (mg/kg):	Click here to enter text.
Route:	Click here to enter text.
When first administered:	Click here to enter text.
Frequency:	Click here to enter text.
Purpose:	Click here to enter text.

Pre-Surgical Procedures and Preparation

11. Sterilization of Instruments (*check all that apply)*

☒Autoclave	Click here to enter text.
☐Chemical Sterilization *(specify agent)*;	Click here to enter text.
☐ Bead sterilization	Click here to enter text.
☐Other *(specify):*	Click here to enter text.

12. Surgeon Preparation for Aseptic Technique *(check all that apply)*	
☐Surgical hand wash	☒Sterile surgical gown
☒Sterile surgical gloves	☐ Surgical Face Mask
☐ Clean Lab Coat *(rats and mice only)*	☐ Surgical Cap/booties
☐ Non-sterile exam gloves *(rats and mice – minor procedures only)*	☐ Other (*list)*: Click here to enter text.

13. Will food be withheld prior to surgery? *(not usually necessary for mice, rats, rabbits)*

☐ No ☒Yes

If **Yes,**

Duration: Up to 18 hours

Justification: Cows should be fasted prior to surgery to reduce rumen volume and potential for rumen contents to spill into the body cavity.

14. Will water be withheld prior to the surgery? *(not usually necessary for mice, rats, rabbits)*

☒No ☐ Yes

If **Yes,**

Duration: Click here to enter text.

Justification: Click here to enter text.

Anesthesia

15. Indicate type of anesthesia that will be used: *(complete the requested information)*

☐Isoflurane

% Induction:	Click here to enter text.
% Maintenance:	Click here to enter text.

☒ Injectable

Drug(s):	Lidocaine 2% paravertebral block
Dose (mg/kg):	100 to 150 mL
Route:	subcutaneous at T13, L1, L2, and L3 and site of surgery
Expected Duration of Agent:	Several hours
Supplemental Dosing information (if needed)	Drug: Xylazine (if needed) Dose: 0.02-0.03 mg/kg Route: IV

16. Monitoring of Depth of Anesthesia *(check all that apply)*

☐ Toe Pinch	☐Tail Pinch
☐ Corneal Reflex	☐ Heart Rate
☒Muscle Relaxation	☐ Respiration Rate
☐EKG	☐ EEG
☐ Mucous membrane color and/or capillary refill time	☒Other (*specify*): pinch area of block

Surgical Procedure

Aseptic Technique must be used on ALL Animals

17. Animal Preparation: (*check all that apply)*

☒Hair Shaved	☒Surgical Scrub

☐ Eye Lubricant	☒Sterile drape
☐ Other (*specify*): Click here to enter text.	
18. Procedure to Maintain Normal Body Temperature: (*check all that apply)*	
☐ Warm Waterbed	☐ Heat pack/pad
☐ Lamp	☐ Reflective Blanket
☒None needed (*explain*): This is not needed in cows undergoing surgery while awake.	
☐ Other (*explain*): Click here to enter text.	
19. Expected Duration of Surgery: **1 hour**	
20. Location and Size of Incision Site(s): **4 inch diameter section of body cavity and rumen removed on the left side of the animal behind the ribs.**	
21. Complete Description of Surgical Procedure *(include sufficient detail that another surgeon could perform the surgery following this description):* The surgery will be conducted by veterinarians from the New Bolton Center. Surgery will be conducted as recommended by the manufacturers of the rumen cannula (http://bardiamond.com/Library/Surgery/Articles/Rumen_Fistula_Surgery-Cattle_Bar_DiamondTM.pdf) and Cornell University (https://ras.research.cornell.edu/care/documents/ACUPs/ACUP207.pdf). The skin will marked at the site of cannula placement and a paravertebral block of T13, L1, L2, and L3 will be done with 2% lidocaine. Skin is removed using a scalpel and the incision then continued through the external oblique muscle. Use forceps to grasp and tent the peritoneum and make an incision into the peritoneal cavity with a scalpel blade to access the rumen. Grasp the rumen with atraumatic tissue forceps and exteriorize the rumen through the incision and secure it to the dermis with stay sutures. Incise the exposed rumen and suture the edges to the skin with size #2 non-absorbable suture. Remove the stay sutures. Place the 3 inch opening cannula into the fistula.	
22. Skin Closure: Click here to enter text.	
☐Wound Clips	☐ Surgical Tissue Glue

☐ Absorbable Suture: Type of suture: Click here to enter text. Size of suture: Click here to enter text.	☒Non-Absorbable Suture: Type of suture: Click here to enter text. Size of suture: #2
☐ None (*explain*): Click here to enter text.	
☐ Other (*explain*): Click here to enter text.	
23. Will Surgical Records be kept? (*Required for USDA covered species*)	
☒ Yes ☐ No	

Post-Surgical Care
Anesthetic Recovery

24. Where will animals be housed during the recovery period?	
☐ OLAM Surgery Suite	☐ OLAM Surgery/Procedure Rooms
☐ OLAM Animal Room (where housed)	☐ OLAM Lab
☒Other *(explain)*: Bedded pack or main herd pen at the University of Delaware dairy farm, 531 S College Ave	
☐ Satellite Lab *(explain)*: Click here to enter text.	
25. Frequency of observation of the animals during recovery:	
☐ Constantly	
☒Periodically (specify period): Cows will be observed hourly for the first three hours after surgery and then at least every 12 hours thereafter when they are retrieved for milking. The surgical site will be observed once daily for the first week after surgery and every other day for the following two weeks.	
26. Procedure to Maintain Normal Body Temperature during Recovery (*check all that apply*):	
☐ Warm air or water bed	☐ Heat pack/pad
☐ Lamp	☐ Reflective Blanket

☒None needed *(explain)*: Not necessary with dairy cattle.	
☐ Other *(explain)*: Click here to enter text.	

Analgesia

27. Procedures/Signs used to assess pain or distress:

Cows will be checked regularly for appetite and attitude. Cows with poor appetite or depressed attitude will be given a physical examination and treated as needed.

28. Analgesic Agent(s): Banamine

	Dose:	1 mg/kg
	Route:	IV
	Treatment schedule:	Immediately following surgery and for 2 days after surgery

Scientific justification for not using analgesia, if applicable:

Click here to enter text.

29. What is the expected time period for complete healing of surgical wounds?

~~After 2 weeks the cannula will be removed. Necrotic tissue and sutures will be removed before replacing the cannula. Complete healing is expected after 4 weeks~~. Complete healing is expected within 3 to 4 weeks. At that time the cannula with the 3-inch opening will be removed and replaced with a cannula with a 4-inch opening. That 4-inch cannula will stay with the animal through the remainder of her life unless it needs to be replaced due to age (typically after 2 or more years).

30. Where will animals be housed during the healing period?

Dairy cow facilities on UD farm

☐ Animal Room

☐ Satellite Lab (*building and room number)*: Click here to enter text.

☒Other (*specify location and explain)*: The main herd pen.

31. Specify the frequency of observation during the healing period:

Cows will be observed at least twice daily for milking. The wound will be observed once daily for the first week following surgery and every other day for the following two weeks.

32. Describe procedures for wound/incision care:

Cows will be caught and placed in a chute once daily for the first week after surgery and every other day for the following two weeks. For the first week after surgery, cows will be given an intramuscular injection of 20 cc ampicillin (Polyflex) daily into the thigh with a 16 gauge, 1.5 inch needle. While in the chute, the cannula will be folded back and the surgery site cleaned with a mild cleansing solution. Necrotic tissue typically sloughs off during this process for the first two weeks following surgery. After each cleaning, a veterinary wound spray (such as AluSpray) will be applied. For the first week following surgery rectal temperature will also be measured. Should abnormal healing or infection be noted at any time, the on-call veterinarian will be contacted.

33. Indicate when wound clips or sutures will be removed, if applicable.

Sutures will be removed 2 weeks following surgery

Additional Information

34. What are the anticipated outcomes of the surgery?

Cows will be fitted with permanent rumen cannulas.

35. How long will the animals be maintained after the surgery?

For their productive life on the dairy.

36. Who will be responsible for post-surgical care?

Richard Morris (UD dairy manager), Tanya Gressley, Amanda Barnard, MacKenzie Conklin

37. Any additional information you wish to include:

Click here to enter text.