The effects of probiotic supplementation on pre and post wean Holstein dairy calf performance and health

By

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TABLE OF CONTENTS

CHAPTER ONE

Review of Literature

LITERATURE REVIEW

INTRODUCTION

During the pre and post wean periods calves are highly susceptible to morbidity and mortality (Hulbert and Moisá, 2016). Morbidity in pre wean heifer calves can impact growth, future reproductive efficiency, and milk production (Abuelo et al., 2021). It has been estimated that mortality in dairy calf rearing operations in the United States is approximately 5% from birth to weaning, with gastrointestinal diseases responsible for the largest proportion. (USDA, 2014).

Calves are born into areas contaminated with bacteria, viruses, and parasites that will affect the newborn calf, which has an immune system that is unprimed without an adequate concentration of antibodies and white blood cells necessary for response to the immunological challenges it will face (McGuirk, 2008). In previous years, antimicrobial use in dairy calves helped mitigate the effects of gastrointestinal infections caused by immunological challenges (Gustafson and Bowen, 1997). However, antibiotic use is only effective against bacterial pathogens, but parasites and viruses, such as *Cryptosporidium* bovine rotavirus and coronavirus, contribute to most gastrointestinal diseases that dairy calves experience in the first 21 days of life (Cho and Yoon, 2014). A calf faces the challenge of developing a structured immune system, establishing a diverse microflora, and initiating ruminal growth within the first few months of life. These health challenges, limitations of antibiotics, and calf development have led to examining other management strategies such as probiotic supplementation to improve calf health and performance.

Probiotics are microorganisms such as lactic acid bacteria (LAB) and *Bacillus* spores, naturally present in the gastrointestinal tract (GIT) and harvested from the soil, that can alter the host's microflora (Vriesman and Benninga, 2021). Previous research has shown that probiotics can aid in developing antimicrobial, antiviral, and antiparasitic responses within the mucosal and systemic immune systems that can affect calf health and can aid in nutrient availability and rumen development that aids rapid adaptation to solid feed (Kayasaki et al., 2021).

Many different bacterial strains have been proposed as probiotics to shift GIT microbial population and thus aid calf health and performance. This literature review will discuss the effect of probiotic species *Bacillus subtilis, Bacillus lichenformis, Lactobacillus animalis,* and *Propionibacterium freudenreichii* on pre and postwean health and performance.

SPECIES AND REPORTED EFFECTS

Bacillus

B. subtilis is a widely studied bacteria that was discovered in 1872. It is a gram-positive, aerobic bacterium in soil and the gastrointestinal tract of ruminants and animals. Mechanisms of its probiotic action are associated with the synthesis of antimicrobial agents such as bacteriocin, increasing non-specific and specific immunity, stimulation of normal microflora of the intestine, and the releasing of digestive enzymes. This probiotic bacterium releases vital digestive enzymes into the intestinal lumen, including amylases, lipases, proteases, and cellulases. These mechanisms of action justify the use of *B. subtilis* as part of a strategy to fight intestinal infections, thus preventing diarrhea and respiratory infections (Savustyanenko, 2016).

B. licheniformis is a gram-positive, endospore-forming organism that can be easily isolated from soil and plant samples. The first published work on *B. licheniformis* was in 1945. It has been shown as a source of antibacterial compounds that inhibit gram-positive pathogens. *B. licheniformis* has been brought into use for livestock to act as a growth promoter, a competitive exclusion agent, to facilitate digestion, and to increase nutrient retention and absorption related to increased fiber digestion in the rumen (Muras et al., 2021).

Calf performance studies have evaluated the effects of *B. subtilis* and *B. licheniformis* on dairy calves. The primary goal of dairy calf raising is to rapidly adapt them to solid feed from a liquid diet. A successful and rapid adaptation to solid feed depends on the development of the ruminal epithelium and ruminal capacity (Krehbiel et al., 2003). Inadequate forestomach development can be the leading cause of health problems and may delay this transition (Beharka et al., 1998). Sun et al. (2011) showed the effects of *B. subtilis natto* on the ruminal development of 24 Holstein bull calves. Calves were enrolled at approximately 7 d of age, given treatment in milk until weaning, and then given treatment in a solid diet. Calves were randomly selected to be slaughtered at weaning or 44 d after weaning to collect rumen fluid for rumen development analysis. The administration of *B. subtilis natto* elevated rumen papillae density and resulted in a larger absorptive surface in the ventral sac. These results suggest that *B. subtilis* natto may accelerate the absorptive potential of end products of fermentation in the rumen of calves. To successfully adapt to solid feed, a calf must maintain a stable intraruminal milieu. The end products of fermentation provide the host with metabolizable energy and regulate rumen pH to stabilize intraruminal milieu. The more rapidly a calf adapts to solid feed, a calf's intake will increase, stimulating rumen muscle and capacity. This research indicates that a probiotic

containing a *B. subtilis* strain may aid in adaptation to solid feed to increase nutrient utilization. This development and successful transition can suggest better intakes at weaning. Higher intakes at weaning have been associated with improved 305-d ME milk, fat, and protein once the calf reaches lactation (Heinrichs and Heinrichs, 2011).

Reported effects have been shown from supplementing a combination of *B. subtilis* and *B. licheniformis*. Showing an effect on reducing gastrointestinal infections in dairy calves is important since neonatal disease will delay subsequent growth (Donovan et al., 1998). Kowalski et al. (2009) determined the efficacy of a feed additive that contained spores of *B. subtilis* and *B. licheniformis* on 64 female Holstein calves. These calves were enrolled at about 2 weeks of age, allocated into a control and probiotic-supplemented group, and monitored up to 8 weeks of age. Probiotic group calves were given treatment in milk replacer and starter mix. Results showed that the probiotic group displayed higher starter consumption (200 g/d higher in probiotic group) and higher ADG than the control group (617 and 668 g/d control and probiotic, respectively) during the whole experiment period. In agreement with this, Bayatkouhsar et al. (2013) showed increased body weight (BW) in probiotic-supplemented calves. This study was one of the few that followed calves past weaning. Twenty-four Holstein heifer calves were supplemented and monitored from 4 to 90 d of age. Ninety-day BW and frame growth measurements, wither height and hip height on probiotic-supplemented calves were higher than control calves. This research may indicate that probiotics caused an improvement in nutrient absorption or a more rapid rumen function development that is important for growth during stressful times like weaning. Successful youngstock growth is vital since higher BW at first calving corresponded with higher

305 d ME fat production on a trial that had a mean age at first calving of 28 mo (Heinrichs and Heinrichs, 2011).

Bacillus-based probiotics have shown no effect on calf growth performance. Riddell et al. (2010) evaluated the effects of *B. subtilis* and *B. licheniformis*, fed in milk replacer, on 40 Holstein calves for the first 56 d of life. Results showed no treatment effect on DMI, ADG, or change in frame growth. The author suggested that the lack of treatment effect may be attributed to the lack of stress the trial calves received while being housed in an indoor and temperature-controlled facility. Probiotics may be more effective when a calf is in higher stress.

Lactobacillus animalis

Lactic acid bacteria (LAB), in general, have been studied for effectiveness as a probiotic in animals since 1965. Its effects are producing pathogen protective compounds such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) that stimulate the host's immune responses (Shida et al., 2006). *In vitro* research on *L. animalis* specifically, showed its ability to survive the GIT, adhere to the mucosa, and produce antimicrobial compounds (Ripamonti et al., 2011).

Successful transition to solid feed depends on the colonization of the intestinal mucosa by ruminal and intestinal microorganisms that prevent the establishment of enteropathogens that lead to diarrhea (Krehbiel et al., 2003). It is vital for the longevity of the calf since morbidity in early life has been correlated to reduced first lactation milk production and lower reproductive

efficiency (Abuelo et al., 2021). Probiotics in the LAB genera have been extensively researched for their ability to promote the colonization of protective bacteria, which competes with pathogenic bacteria, thus counteracting the adverse effects of diseases in calves. Agazzi et al. (2014) evaluated the effects of LAB on 22 female Holstein calves, starting at 2 d of age and continuing to 28 d of age. Veal calf feces were sampled, and the most frequent LAB that occurred within the samples were isolated and used to create a species-specific probiotic that included *L. animalis, L. paracasei,* and *B. coagulans.* This study evaluated fecal LAB/pathogenic *E. Coli* ratio, which the author used as a parameter to evaluate the calf GIT microbial health. Results showed higher LAB/ pathogenic *E. Coli* ratio in probiotic supplemented calves compared to control calves. This indicates an increase in non-pathogenic bacteria, leading to competition between pathogenic and non-pathogenic bacteria, thus reducing the incidence of calf scours and mortality. This suggested a favorable equilibrium in the microbiota that was confirmed by a lowered frequency of diarrhea in the LAB group (63.3%) compared to the control group (70.7%). In agreement with this, *in vitro* research has shown the activity of LAB and its influence on protection from neonatal gastroenteritis caused by viral pathogens, including bovine respiratory disease, bovine coronavirus, and bovine viral diarrhea, which is the main cause of morbidity and mortality in the early stages of claves which lead to large economic losses to producers (Timmerman et al., 2005; Aich et al., 2007). LAB stimulated the expression of antiviral proteins capable of inhibiting and removing viral pathogens (Chiba et al., 2012). These findings indicate that LAB can aid in balancing the microflora, which maintains the host's health and thus nutrient absorption.

In contrast, other research has shown no effect of LAB on calf health. Jenny et al. (1991) observed the effects of 2 species of *lactobacillus* and *B. subtilis* on 84 Holstein calves from 3 to 30 d of age. Health results showed no treatment effect on fecal consistency, days with scours, or average body temperature. The author suggested that no effect was shown since calves were overall healthy and that probiotics show an effect when calves are overall unhealthy and have less diverse microflora. No effect may be attributed to the calves remaining with the dam for 2 d postpartum, where all calves may be exposed to more microflora compared to sterilized bottles. There is no evidence of initial serum total protein being recorded in Jenny et al. (1991).

Probiotics from these species have been shown to affect rumen function through the improvement of feed efficiency (FE). A meta-analysis conducted by Frizzo et al. (2011) evaluated 14 studies of calves supplemented LAB and its effect on FE. Overall, LAB probiotics were found to improve FE. However, when studies were partitioned into feed types, calves fed whole milk showed no effect while calves fed milk replacer showed a positive effect of probiotics. Whole milk has the potential to contain a level of bacterial flora that may diminish the effects of a probiotic specified for the probiotic group.

Propionibacterium freudenreichii

There has been evidence that the *propionibacteria* species have probiotic capabilities stemming from their ability to form propionic acid and act as growth stimulators for other beneficial bacteria in the GIT. *P. freudenreichii*, specifically, was found as the most effective on health and

performance of the *propionibacteria* genera when supplemented to young piglets (Mantere-Alhonen, 1995).

Fujisawa et al. (2010) evaluated the protective effects of *P. freudenreichii*, which is a species far less researched in calves than LAB or *Bacillus* strains. Twelve male calves were fed a probiotic in milk replacer containing *L. gasseri* and *P. freudenreichii* from 4 to 42 d of age. Fecal samples were collected at 21 and 42 d of age. Fecal concentration of *Bifidobacterium* was higher in the probiotic treated group. This was used as an indicator for microflora condition because *Bifidobacterium* concentration has been reported to be closely related to clinical symptoms, and an increased population has shown anti-scouring effects in early-weaned calves (Kimura et al., 1983). This was confirmed by the fact that the probiotic-fed group had more calves with normal fecal water content and normal fecal scores than the control group. These results suggest that a product containing *P. freudenreichii* may aid in the protection of the GIT of a young calf.

PERFORMANCE ON A COMMERCIAL DAIRY

Much of the previous research in probiotics has been done with small sample sizes, however, to validate its use in a practical setting, it is essential to evaluate the effects of these species on a commercial dairy with a larger sample size and a more variable environment. Lucey et al. (2021) observed the effects of yeast culture enriched with mannan-oligosaccharide prebiotic, *B. Subtilis* and *Lactobacillus plantarum* probiotic, and a combination of both on 1,801 Holstein heifer calves in a commercial herd. These calves were enrolled at birth and delivered the treatment in milk until weaning. The probiotic failed to demonstrate a beneficial effect on BW gain compared

to the control group. This result may be attributed to a milk replacer blended with whole milk being fed to all treatment groups. As stated before, whole milk has the potential to contain a level of bacterial flora that may diminish the effects of a probiotic specified for one group. It was found that the probiotic group showed a decreased number of *Cryptosporidium* oocysts in the 14 d fecal collection. This may show that *B. subtilis* has the potential to reduce the environmental burden of a parasite that can cause calf diarrhea and death, leading to economic losses for the calf-raising facility.

CONCLUSION

B. subtilis, B. lichenformis, L. animalis, and *P. freudenreichii* have shown mixed results in their effectiveness in lowering disease occurrence and improving fecal condition, ADG, frame growth, FE, and DMI. most of the dairy calf research has been conducted on a small scale and evaluates effectiveness only in the prewean period. Research evaluating these species of probiotics conducted on a larger scale and that monitors calves into the postwean period can be advantageous for calf management practices.

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KEYWORDS: Dairy Calves, Probiotics, Diarrhea

TABLE OF CONTENTS

CHAPTER TWO

The effects of probiotic supplementation on pre and post wean Holstein dairy calf performance and health

INTERPRETIVE SUMMARY

Since neonatal calves are highly susceptible to morbidity that can negatively impact growth and future performance, the dairy industry has looked to probiotics for protection against disease. This study supplemented a multi-strain probiotic fed from birth to 180 d to dairy calves on a commercial dairy farm. No overall difference in health and performance outcomes were found in calves fed this probiotic compared to controls.

ABSTRACT

Feeding probiotics has been shown to improve the health and production characteristics of dairy calves. The objective of this trial was to evaluate the effect of a probiotic fed daily on health outcomes, average daily gain, frame growth, feed efficiency, and intake when fed to Holstein heifers from birth to 180 d. A total of 324 Holstein heifer calves from a California dairy were enrolled within 48 h of birth into two treatment groups: 1) control group (CON: 176 calves) were given 0.5 g of lactose added to milk once daily from birth to weaning (60 d) and then 0.75 g in grain from weaning to 180 d and 2) probiotic group (PRO: 153 calves) were given 0.5 g (1.1×10^{10} CFU^2/g) of probiotic in milk once daily from birth to weaning and then 0.75 g (1.65×10^{10} CFU^2/g) of probiotic in grain from weaning to 180 d of a *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii* probiotic (Bovamine Dairy Plus, Chr. Hansen, Milwaukee, WI). The lactose powder was given to CON to balance the lactose content in the probiotic treatment and to ensure no difference in the handling of milk bottles. The PRO ADG was lower during the hutch period (1 - 91 d) and higher fecal shedding of *Clostridium* at 21 d and 42 d collections. There were no differences in all other health and performance outcomes.

INTRODUCTION

A dairy calf is highly susceptible to morbidity and mortality during the pre and postwean periods, which will impact the future success of a herd (Hulbert and Moisá, 2016). Calf mortality has been estimated to be 5% from birth to weaning in the United States. For heifers that survive past weaning but had a morbidity event, subsequent growth, future reproductive efficiency, and future milk production could be negatively affected (USDA, 2014; Abuelo et al., 2021).

Antibiotic use in dairy calves has helped mitigate the effects of gastrointestinal infections caused by immunological challenges (Gustafson and Bowen, 1997). However, antibiotic use is only effective against bacterial pathogens. Parasites, such as *Cryptosporidium,* and viruses, such as bovine rotavirus and coronavirus, contribute to most gastrointestinal diseases dairy calves experience in the first 21 days of life (Cho and Yoon, 2014). Probiotics have mitigated the effects of pathogenic bacteria, parasites, and viruses (Kayasaki et al., 2021). Supplementation of probiotics such as lactic acid bacteria (LAB), *Bacillus* spores, and *P. freudenreichii* has been used in calves to shift GIT microbial populations to enhance antimicrobial, antiviral, and antiparasitic responses within the mucosal and systemic immune systems. Agazzi et al. (2014) evaluated the effects of administered LAB and had a lowered frequency of diarrhea in the LAB group compared to the control when fed to 22 female Holstein calves, starting at 2 d of age and continuing to 28 d of age. Fujisawa et al. (2010) also evaluated the protective effects of *P. freudenreichii*. Twelve male calves were supplemented with either probiotic or control from 4 to 42 d of age. The probiotic-fed group had more calves with normal fecal water content and normal fecal scores than the control group.

Growth and intake outcomes have improved with probiotic supplementation, but results have been inconsistent. Kowalski et al. (2009) supplemented a two-species probiotic that contained *B. subtilis* and *B. licheniformis* and a control to 64 female Holstein calves fed from 2 to 8 wk of age. The probiotic group displayed higher starter consumption and higher ADG than the control group. In contrast, Riddell et al. (2010) evaluated the effects of an identical two-species probiotic for the first 56 d of life on 40 Holstein calves. There was no treatment effect on DMI, ADG, or change in frame growth. The author suggested that no effect of treatment may be due to the controlled environment, indoor and temperature-controlled, used in the study.

Much of the previous probiotic research has been done with small university herds. However, to validate its use in a practical setting, it is essential to evaluate the effects of these species on a commercial dairy. Lucey et al. (2021) observed the effects of yeast culture enriched with mannan-oligosaccharide (MOS) prebiotic, *B. subtilis,* and *Lactobacillus plantarum* probiotic on 1,801 Holstein heifer calves in a commercial herd. These calves were enrolled at birth and were delivered the treatment in milk until weaning. Overall, ADG was higher in the MOS and probiotic treatment than control.

The effectiveness of combining species of microbes and administering probiotics past the weaning period has not been extensively studied on a commercial dairy. Therefore, the objective of this study was to evaluate the effect of a probiotic blend of *B. subtilis, B. lichenformis, L. animalis,* and *P. freudenreichii* fed daily on health outcomes, fecal consistency and pathogen shedding, ADG, frame growth, feed efficiency, and intake when fed to dairy heifer calves from birth to 180 days of age. Our hypothesis was that health and performance outcomes on a

commercial dairy would be improved when calves were fed probiotics compared to control from birth to 180 d.

MATERIALS AND METHODS

All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee (protocol 22245).

Study design and population

Holstein heifer calves were enrolled in a block randomized trial on a 5,500-milking-cow Holstein dairy in Kings County, California between October 4 and December 6, 2021. Calves were blocked by row and assigned to treatment based on birth order. Calves that were assessed as severely ill at birth by the farm staff were excluded from enrollment. Treatment groups: 1) control group (CON: 176 calves) were administered 0.5 g of lactose in milk once per d from birth to weaning (60 d) and then 0.75 g in grain from weaning to 180 d and 2) probiotic group (PRO: 153 calves) were administered 0.5 g (1.1×10^{10} CFU^2/g) of probiotic in milk once per d from birth to weaning and then 0.75 g (1.65×10^{10} CFU^2/g) of probiotic in grain from weaning to 180 d. The probiotic consisted of *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii* (Bovamine Dairy Plus, Chr. Hansen, Milwaukee, WI). The lactose powder was given to CON to balance the lactose content in the probiotic treatment and to ensure that all bottles were handled similarly. A subset of 60 calves from both CON and PRO were selected for fecal collection and intake measurements.

Calf management, housing, and feeding in hutches

In the maternity pen, calves received 4 L of colostrum within 30 min of birth and 2 L at 10 h. Calves were moved from the maternity area to individual hutches within 24 h of birth. Hutches were metal with wire sides 1 m wide, a tin shade, and almond shell and rice hull bedding. Hutches were in rows of 60 with 0.5 m of space between each hutch. Ad libitum water was available to all calves.

From 1 – 21 d, calves were fed 3 L of milk replacer powder (Plasma 26:20 P, American Calf Products, Turlock, CA). From 22 – 44 d, calves were fed 3 L of a combination of hospital milk with milk replacer fed twice daily at 0600 h and 1300 h. Calves 45 – 52 d were reduced to 2 L twice daily of hospital milk with milk replacer powder fed at 0700 h and 1400 h, and from 53 - 60 d, calves were reduced to 1 feeding of 2 L of hospital milk with milk replacer powder once a d at 0700 h. Treatments were prepared by dissolving 0.5 g and 0.75 g for prewean and postwean calves, respectively, of PRO (*B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii)* or CON (lactose powder) into 5 mL of deionized water . During the prewean period, treatments were administered into their respective milk bottles via syringe, mixed gently, and delivered to calves. Pelleted calf starter grain was offered ad libitum from 1 – 45 d at 0700 h daily. Calves were transitioned to the next grain mix using a blend of the pelleted starter and a textured grain mix over a week, and then fed only a textured grain mix ad libitum at 46 - 90 d, 0900 h daily. After weaning, treatments were applied as a top dress via syringe into their respective feed buckets immediately after fresh grain mix was added.

Calf management, housing, and feeding in corrals

At 90 d of age, heifers were moved to small dry lot corrals with a flush lane at the feed bunk, shade, water troughs, and headlocks. The corrals held 14 heifers and heifers were sorted into pens of the same treatments as previous. The heifers remained in these pens for 40 d and then at 130 d they were moved to a larger, dry lot pen of 50 heifers.

In the small pens, heifers were fed the textured grain mix at 0745 h. Once moved into the large corrals, heifers transitioned from the textured grain mix to a forage-based TMR for 1 wk. The heifers were then only fed the TMR, which was given at 0500 h daily until the end of the trial. At all feedings, heifers were locked out of the feed bunk, treatments were top-dressed onto fresh feed to their respective pens via the 0.75 g / 5 mL dilution syringe, and the headlocks were opened for the calves to eat.

Milk and feed sample collection

Milk samples were collected from the pasteurized hospital milk, the blend of pasteurized hospital milk and milk replacer weekly from bottles during the morning feeding. These samples were stored in a 3°C refrigerator and were analyzed every 2 wk for nutrient composition by Tulare Co. DHIA. All feed samples were collected weekly using the hand-grab method (Robinson et al., 1998). These samples were stored in a -20°C freezer, weekly feed samples were pooled by weight for each month and sent for nutritional analysis to Analab (Analab, Fulton, IL).

Assessment of passive transfer

Blood samples were collected from all calves between 24 and 48 h of birth by jugular venipuncture using a 10-mL red-top serum tube (BD Vacutainer, Fraklin Lakes, NJ). Blood

samples were centrifuged within 3 h of collection for 15 min and measured for serum total protein (TP) using a handheld Refractometer (Brix Refractometer, ADE Advanced Optics, Oregon City, OR) to assess passive transfer. A TP categorization was adapted by Lombard et al. (2020) and was used to assess passive transfer. The categories were: ≥ 6.2m g/dl, excellent; 5.8 - 6.1 g/dl, good; 5.1 - 5.7 g/dl, fair; < 5.1, poor.

Weight, hip, and shoulder height measurement

Heifer BW, hip height (HH) and shoulder height (SH) were recorded at enrollment, upon exiting the hutch, and at completion of the trial at 180 d of age. Body weight was measured using a digital platform scale (Tru-Test, Mineral Wells, TX) and HH and SH were measured using a measuring stick (Nasco, Fort Atkinson, WI). The hutch period growth measurements were calculated as the difference in each measurement between the first and second measurement (1 - 91 d). The pen period growth measurements were calculated as the differences between the second and third measurement (92 - 180 d). Lastly, the overall growth period was calculated as differences between the first and third measurement (1 - 180 d).

Health and fecal scoring

Health and fecal scoring were performed once daily while calves were in hutches after the morning feeding. Health scoring was done on a 1 to 5 scale adapted from Sayers et al. (2016) that evaluated demeanor, mobility, hydration level and ears, with 1 being clinically normal and 5 being gravely ill. A clinical normal calf (score = 1) would show signs of bright, alert, responsive demeanor, active, and clear bright eyes. A calf given a mild health score (score = 2) would show signs of a dull and fairly responsive demeanor, ears slightly drooped, and eyes slightly sunken. A

calf given a severe health score (score ≥ 3) would show unresponsiveness, drooped ears, difficulty standing, and sunken eyes. Length of first health event was calculated as days between the onset and end of an episode of a 2 or greater health score. Fecal scoring was based on fecal consistency and was evaluated by observing the freshest feces visible on the hutch flooring. A fecal score of 1 to 3 was adapted from Lucey et al. (2021), with 1 being normal, 2 being firm and semi-loose, and 3 being watery. Length of diarrhea was calculated as days between the onset and end of an episode of diarrhea.

Fecal collection and analysis

Fecal samples were collected from each enrolled calf on 7, 14, 21, and 42 d of age to enumerate the average number of colony-forming units per gram of fecal sample (CFU/g) of three bacterial types; *Escherichia coli*, Shiga toxin-producing *E. coli* (STEC), and *Clostridium perfringens*. Fecal samples were collected after morning feeding, with at least 10 g collected from each calf by digital manipulation using lubricated, gloved hands. Fecal samples were collected into sterile 50 mL tubes and immediately placed in a cool box with ice packs for transportation to the laboratory. In the lab, 7 g of each fecal sample was homogenized in a 40% glycerol/TRIS solution. A subsample of 1.6 mL of feces/glycerol homogenate was transferred to a 2.0 mL Eppendorf tube for storage at -80°C for later analysis. For plating, samples were thawed overnight in a fridge and kept at 5°C to prevent bacterial replication. For the first dilution (10x), the entire vial content (0.8 g feces in 1.6 mL volume homogenate) was transferred into a 15 mL culture tube containing 6.4mL of 1x Phosphate-buffered saline (PBS) to make a 10-fold dilution. Subsequent dilutions were made by transferring 1 mL of the previous into 9 mL of 1xPBS in a culture tube. Each sample was then plated using WASP spiral plater (Microbiology International,

Fredrick, MD) on selective media specific for each bacterial type; CHROMagar™ *E. coli*, CHROMagar™ STEC, and CHROMagar™ *C. perfringens* for *E. coli*, STEC, and *C. perfringens*, respectively. Control plates (CON) comprised of 10-fold serial dilutions of reference strain cultures starting at $1x10^8$ concentration (MacFarland standard estimate). Control plating was performed on each sample plating day to check for quality control. Plated samples were incubated at 37°C for 18 - 24 under aerobic (*E. coli* and STEC) and anaerobic (*C. perfringens*) conditions. The CFU/g for each plate was counted using SphereFlash automatic colony counter (Neutec, Farmingdale, NY). An enlarged image of each plate was observed to check for any errors in the SphereFlash identification of colonies, and data were exported to an Excel spreadsheet for statistical analysis.

The Kinyoun cold stain is used to demonstrate *Cryptosporidium* in direct smears of fecal material. Smears were dried, heat-fixed, and then flooded with a Kinyoun carbol-fuchsin. Slides of smears were rinsed, decolorized with 1 - 3 % sulfuric acid, and rinsed again. Slides were then counterstained with brilliant green or methylene blue for approximately 1 min. Lastly, slides were rinsed with tap water, air dried, then observed using 40X or l00X objectives. *Cryptosporidium* oocysts were evaluated categorically as the presence of oocysts per field on the slide (none, rare, small, moderate, or large).

Intake observation

Individual intakes were estimated at 21, 42, 60, and 74 d of age by filling 19 L buckets with 3 d of fresh solid feed, weighed, and placed next to the calves of interest. These calves had grain placed in their hutch grain bucket daily via the calf's assigned 19 L bucket. After 3 d, any

refused feed from the hutch grain bucket was placed back into the assigned 19 L bucket and weighed. The difference between the feed weight prior to the measurement period and refused feed was divided by the number of days of feeding (3) and used as daily estimated feed intake.

While in corrals, daily intake was measured on a pen basis and averaged by calf at 91, 112, 133, 157, and 175 d. All pens' feed lanes were cleaned before the feed was dropped. For the smaller pens, the total weight of feed was estimated by observing the weight on the feed wagon scale just before and after dropping the feed in front of an individual pen. For the large pens, the total weight dropped was collected from the feed management software EZFeed (DHI-Provo, Provo, UT). After 24 h, the feed was collected and weighed to determine refusal weight. The difference between total feed dropped, and refusal weight was divided by the number of heifers in the pen that day to estimate 24 h individual calf intake for that pen.

Farm treatment records collection

Diagnosis and treatment of sick calves were carried out by farm staff as per farm protocol. Calves were evaluated for signs of clinical disease and treated once daily by farm staff. Farm treatment records for all treatments administered and diagnoses of health conditions were extracted from the farm herd management software (Dairy Comp 305, VAS, Tulare, CA). Scours were treated with ceftiofur and flunixin. Pneumonia was treated with enrofloxacin. Scour and pneumonia events were defined as a calf having any record of antibiotic treatment for scours or pneumonia per the farm treatment record.

Power analysis

To detect differences in ADG (g/d) between CON and PRO pre-wean calves, a two-tailed power analysis was run using SAS (SAS Institute v. 9.4, 2021) assuming a normal distribution with an alpha of 0.05 and statistical power of 0.8. Control ADG was assumed to be 900 g/d with SD = 31 (Lucey et al., 2021) with a change in ADG of 15 g for a resulting sample size of at least 140 calves per PRO and CON treatment.

Statistical analysis

The unit of interest was individual calf for health, growth, fecal shedding of bacteria, and intake in hutches. The unit of interest was pen for intake outcomes in corrals. For intake in pens, there were 6 replicates for each treatment. Data analyses were performed using SAS (SAS Institute v. 9.4, 2021). Statistical differences were considered as $P < 0.05$. Baseline characteristics were evaluated to test for successful randomization using one-way ANOVA for initial TP (g/dL), 1 wk average milk intake, BW, HH, and SH using PROC ANOVA in SAS.

Growth performance, health outcomes, and intakes in the hutch and pen were evaluated using linear regression (PROC GLM, SAS v. 9.4). For all outcomes, linear regression models were constructed and then assessed for normality and homoscedasticity of residuals. For nonnormal residuals, the dependent variable length of first diarrhea event were log-transformed. Independent variables were retained in the model if $P < 0.05$. The independent variables for growth outcomes were treatment, row, TP, birth BW, birth HH, age in days at outcome measurement. Dependent variables were BW gain, ADG, HH, SH, and frame growth where frame growth = $(\Delta HH + \Delta SH)/2$. The independent variables for intake outcomes were treatment, row, pen and birth BW. Dependent variables were average intake and feed to BW gain (F:G).

The independent variables for health outcomes were treatment and row. Dependent variables were fecal counts of *Escherichia coli*, *Escherichia coli* (STEC), and *Clostridium*, length of first diarrhea (d), length of first health event (d), total days with diarrhea (fecal score = 3), total days with a mild health score (health score = 2), and total days with a severe health score (health score ≥ 3).

A Poisson regression model with robust standard errors (Zou, 2004; PROC GENMOD, SAS v. 9.4) was used to identify differences in presence of *Cryptosporidium* oocysts, risk of removal from trial by being identified by farm staff as having severe clinical illness, mortality, at least one event of diarrhea, mild health, severe health, and at least one farm-recorded event of scours and pneumonia. The dependent variable for the model was number of days and independent variables treatment, row and TP. Row and TP were retained in the model since the variables *P*-values were < 0.05.

A Kaplan-Meier analysis (PROC LIFETEST, SAS v. 9.4) was used to compare the median days to first diagnosed diarrhea and compute the Kaplan-Meier curve, a nonparametric maximum likelihood estimate. This survival analysis of time to first diarrhea was conducted to evaluate if probiotics can potentially delay the time to diarrhea, as the timing of this event can affect performance in the early stages. Log-rank tests for homogeneity were used to indicate the difference between PRO and CON.

RESULTS AND DISCUSSION

Baseline comparison

Diet analyses were performed to ensure that PRO and CON received consistent nutrient composition throughout the trial (Table 1). The standard deviations of most nutrient composition variables were low relative to the means. Fat percentage in the milk replacer and ash in the textured grain mix had relatively high standard deviations compared to the other composition variables. However, the low variation in most variables indicates that the calves in this trial received consistent nutrient supplies.

Initial measurements of TP, HH, SH, BW, and milk intake (1 wk avg.) were not different between the treatments indicating that the treatment groups were uniform at baseline. (Table 2). Initial measurements TP, HH, and BW were used as covariates in the analysis for all subsequent measurements.

Diarrhea and health scores

Diarrhea outcomes (Tables 3 and 4) during the first 4 wk of life was the period with the highest incidence of diarrhea. Length of diarrhea outcome was used to determine if the probiotic could shorten a diarrhea event. However, there was no difference between PRO and CON length of first diarrhea event. As shown in the Kaplan-Meier survival analysis (Figure 1), the probiotic did not delay the time to first diarrhea event. In total, 309 calves experienced diarrhea and were included as failure events. The median days to diarrhea were 9 d and did not differ among treatments. A Log-Rank model for first diarrhea hazard was conducted and showed no difference in the hazard of diarrhea between treatments. Jenny et al. (1991) observed the effects of 2 species of *lactobacillus* and *B. subtilis* supplemented from 3 to 30 d of age. Health results showed no treatment effect on fecal consistency or days with scours, similar to this trial.

The incidence risk of diarrhea was calculated to evaluate diarrhea prevention, and there was no treatment effect of the risk of at least one diarrhea event (Table 4). However, PRO had a greater number of days with a fecal score of 3 than CON (Table 3). The difference in days with a fecal score of 3 between PRO and CON was under 1 d and may not provide a large enough time frame to detect different amounts of scours treatments. This is supported by the finding that there was no difference in the risk of being treated for scours (Table 4).

Similarly, days with health score ≥ 3 were higher in the PRO than the CON (Table 3). The difference was 0.2 d. This difference would not be detectable since health scores were only evaluated once a d. Previous studies that have looked at the effect of probiotics on health have shown the benefits of probiotics using internal markers, such as increased cytokine concentration and macrophage development (Sun et al., 2010; Xu et al., 2012). However, measurable observations, such as those collected in this trial, will impact a heifer's future success in reproduction and milk production (Heinrichs and Heinrichs, 2011) and so provide valuable evidence of the success or failure of probiotic supplementation.

Pathogen fecal shedding

There were no differences in CFU/g of *Escherichia coli*, *Escherichia coli* (STEC), and day 7 and 14 *Clostridium* (Table 5). The results in this trial were similar to Górka et al. (2021). The Górka trial supplemented a *B. licheniformis* and *B. subtilis* probiotic to 64 Holstein calves from 10 to 60 d, and there was no difference in fecal shedding of pathogens. Unfortunately, due to issues with sample storage, the PRO had less than half of the samples analyzed than the number of calves in

the subset. However, the sample size was large enough to show a difference between PRO and CON in 21 d and 42 d *Clostridium*. Fecal shedding of *Clostridium* was higher in PRO compared to CON at 21 d and 42 d.

The parasite *Cryptosporidium* was evaluated by the Kinyoun cold stain method (Table 6). There were so few samples in the large or moderate categories that *Cryptosporidium* was evaluated as no presence or any presence of oocysts. There was no difference in risk of shedding *Cryptosporidium* oocysts. Harp et al. (1996) measured Cryptosporidium oocysts shedding on a commercial dairy in Holstein calves fed a LAB probiotic or a control treatment for the first 10 d of life. There was no treatment effect in the shedding of oocysts in the Harp trial. In Harp et al. (1996), oocysts were present in more than 80 % of the calves in all treatment groups. In the current trial, less than 40 % of all calves had oocysts, which includes calves with a very low presence of oocysts. *Cryptosporidium* was only assessed by the Kinyoun cold stain method, which is less sensitive and specific than other methods (Weber et al., 1991). Although a different *Cryptosporidium* detection method, this parasite appeared to have a lower presence in the calves that were enrolled in this trial. There may not have been enough of a challenge to detect an effect of a probiotic.

Intake and feed efficiency

There were no differences in mixed grain intake at all time points in the hutch or pen intake between PRO and CON (Table 2). This finding was similar to a meta-analysis by Alawneh et al. (2020), which showed no difference in DMI when feeding probiotics compared to controls using results from 32 calf trials. All trials in the meta-analysis observed intake during the prewean

period (at most 9 wks), equivalent to the hutch period in this trial. There was also no difference in F:G ratio in the hutch. Kekana et al. (2020) supplemented a LAB probiotic blend from 4 - 42 d to 32 Holstein calves and observed no difference in feed efficiency. Hospital milk fed during the trial may have mitigated the effects of this probiotic on feed intake and feed efficiency. From 1 - 21 d, calves were fed milk replacer. However, between 22 - 60 d, milk feeding was a combination of pasteurized hospital milk supplemented with a milk replacer. A meta-analysis by Frizzo et al. (2011) examined the effects of probiotics on feed intake and feed efficiency in prewean calves. In this meta-analysis, when studies were separated into feed types. When calves were fed milk replacer, probiotics positively affected feed efficiency. But probiotics fed to calves given whole milk had no effect on feed efficiency.

Growth in hutches

Most trials that have evaluated probiotics in calves have either reported no difference or a positive effect of probiotics on growth. In this study, PRO had lower BW and lower ADG than CON during the hutch period (Table 2). Lucey et al. (2021) showed that BW gain was lower in the probiotic group (fed *B. subtilis*) compared to control in calves 7 - 42 d. Higher BW gain in the CON versus the PRO could also be due to higher *Clostridium* shedding at 21 d and 42 d in PRO than CON (Table 5), although there was little difference in fecal and health scores (Table 3). Nutrient supply may have been diverted in the PRO to combat disease rather than put towards growth (Carroll and Forsberg, 2007).

There were no differences in SH, HH, and frame growth during the hutch period (Table 2). Riddell et al. (2010), who fed a similar probiotic blend on 40 Holstein calves for the first 56 d of

life, found no difference in frame growth measurements. They suggested that probiotics may be more effective when a calf is under stress, and studies with calves at well-managed facilities might not be affected. During the hutch period in this study, there was a greater BW gain in the CON group. This may suggest that the CON calves gained BW that did not contribute to their frame size.

Growth in pens

There were no differences in ADG and BW gain in the pen period and overall measurements between Con and PRO (Table 2). The lack of difference in the current trial may be due to an increase in variation of measurements in the postwean compared to prewean period. For BW gain, the SE in the hutch period was 16, and the SE in the pen period was 21. As the population of calves became older, there was a greater variation in BW gain. Soltan (2009) also had increased variation from prewean to postwean period. Although the Soltan trial was not a probiotic trial, BW gain SE in the prewean period from 1 - 8 wk was 1, and SE in the postwean period from 9 - 24 wk was 5. Since variation was higher as calves grew older, the product would need more of an effect or an increase in sample size to detect an effect due to the probiotic. Since there was a lower BW gain in the PRO during the hutch period and no difference in the pen period, there may have been a dilution that explains no difference in BW gain in the overall period.

Established microflora

During this trial, most outcomes in health, growth, and fecal pathogens, and all outcomes in intake and feed efficiency, were not different between PRO and CON. Previous studies with no

effects of probiotics on growth and efficiency outcomes have stated that probiotics may not have an effect on healthy calves (Jenny et al., 1991; Riddell et al., 2010). The Dairy Calf and Heifer Association publishes benchmarks for calf management goals (DCHA, 2016). Scours treatments and mortality on the facility where this trial was conducted were well below the benchmark stated by DCHA. This suggests that the facility in which this trial was conducted was well managed. In addition, the USDA National Animal Health Monitoring System's Dairy 2014 study created four categories ranging from excellent to poor (Lombard et al., 2020). These categories were divided by thresholds in which certain TP levels corresponding to changes in the future success of the calf. The higher the TP category, the less likely a calf would be susceptible to morbidity and mortality. In this study, baseline TP levels were high for both treatment groups (Table 2), placing both groups well over the threshold for the excellent category. This suggests that the calves were well prepared for encountering stressful periods such as weaning. The microflora provided in the probiotic may not alter an already well-established gastrointestinal tract microbial population.

CONCLUSION

There were few differences between CON and PRO treatment results. The PRO had a lower ADG in the hutch and higher fecal shedding of *Clostridium* at the 21 and 42 d collections. There were no differences in all other health and performance outcomes.

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Table 1. Nutrient and chemical composition of the diets

Item	n[1]	Mean	SD
Milk replacer[2], % As-Fed	8		
Fat		2.17	0.84
Pro		3.75	0.18
Lac		5.72	0.16
SNF		10.47	0.32
Milk replacer with PHM[3], % As-Fed	15		
Fat		3.62	0.45
Pro		3.92	0.16
Lac		5.64	0.28
SNF		10.54	0.44
Starter pellet[4], % DM	3		
DM, %		85.70	0.24
CP		23.89	0.46
NDF		21.15	0.29
Fat		4.22	0.28
Starch		27.48	0.34
ASH		6.57	0.22
NFC		46.42	0.35
Ca		1.03	0.16
P		0.56	0.04
Na		0.29	0.08
K		1.29	0.06
Textured grain Mix[5], % DM	5		
DM, %		84.83	0.68
CP		17.73	1.32
NDF		27.04	5.59
Fat		3.31	0.52
Starch		37.37	1.71
ASH		3.49	1.15
NFC		51.50	5.59
Ca		0.86	0.05
P		0.44	0.02
Na		0.27	0.07
K		0.80	0.01
TMR[6], % DM	3		
DM, %		57.09	2.16
CP		19.53	0.04
NDF		28.50	1.04
Fat		2.78	0.15
Starch		25.04	1.51
ASH		8.07	0.20
NFC		43.27	1.10

Ca	1.56	0.02
P	0.48	0.01
Na	0.20	0.04

[1]n = samples analyzed
[2]Milk replacer (1 - 21 d)
[3]Pasturized hospital milk (PHM) blend with milk replacer (22 - 60 d)
[4]Starter pellet (1-45 d)
[5]Textured grain mix (46-130 d)
[6]TMR (131-180 d)

Table 2. LSM comparison of baseline and growth measurements by treatment

	Treatment[1]			
Item	CON	PRO	SE	*P*-Value
Baseline Measurements				
Calves enrolled	176	153		
Serum total protein, g/dL	7.2	7.3	0.1	0.2
BW, kg	39	39	0.5	0.2
Shoulder height, cm	73.5	73.5	0.5	0.4
Hip height, cm	77.5	78	0.5	0.3
Milk intake (1 wk avg.), L	5.6	5.6	0.1	0.2
Hutch period (1 - 91 d)				
Calves included in model	170	148		
ADG, g/d	774	729	16	< 0.05
BW gain, kg	69	65	1.5	< 0.05
Shoulder height gain, cm	17	17	0.5	0.6
Hip height gain, cm	20	20	0.3	0.9
Frame growth[2], cm	18.5	18.5	0.4	0.5
Intake[3], kg	1.9	1.9	0.1	0.7
F:G[4], kg/kg	2	2	0.1	0.6
Pen period (92 - 180 d)				
Calves included in model	167	143		
ADG, g/d	1106	1106	21	0.9
BW gain, kg	99	101	2	0.2
Shoulder height gain, cm	19.5	20	0.5	0.4
Hip height gain, cm	19.5	20	0.5	0.2
Frame growth[2], cm	19.5	20	0.5	0.2
Intake[5], kg	12.6	13.7	0.9	0.2
Overall period (1 - 180 d)				
Calves included in model	167	143		
ADG, g/d	938	924	15	0.3
BW gain, kg	167	166	2.5	0.6
Shoulder height gain, cm	37	36.5	0.5	0.4
Hip height gain, cm	39.5	39	0.5	0.6
Frame growth[2], cm	38.5	38	0.5	0.4

[1]Treatment groups included a control (CON; Lactose) or probiotic (PRO; Lactose based *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii*)
[2]Frame growth: Difference of average hip and shoulder height between listed days
[3]Average intake of observations at 21 d, 42 d, 60 d and 74 d
[4]Total kg of feed consumed divided by total kg of weight gained
[5]Average intake of observations at 91 d, 112 d, 133 d, 157 d and 175 d

Table 3. Effect of probiotic on diarrhea, and general health scores in prewean period

Item	n[2]	Treatment[1]		SEM	*P*-Value
		CON	PRO		
Diarrhea					
Days with fecal score of 3, d[3]	313	3.1	3.6	0.2	< 0.05
Length of first diarrhea event, d[4]	313	4.7	5	0.2	0.2
Health					
Days with health score of 2, d[5]	324	7.7	8.2	0.7	0.1
Days with health score ≥ 3, d[6]	319	0.2	0.4	0.1	< 0.05
Length of first health event, d[7]	318	4.2	4.8	0.4	0.1

[1]Treatment groups included a control (CON; Lactose) or probiotic (PRO; Lactose based *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii*)
[2]n = number of calves in the model
[3]Loose and watery feces observation (1 - 28 d)
[4]First event of at least 3 consecutive days with fecal score 2 or greater (1 - 28 d)
[5]Observed dull and fairly responsive demeanor, ears slightly drooped, and eyes slightly sunken (1 - 59 d)
[6]Observed unresponsiveness, drooped ears, difficulty standing, and sunken eyes (1 - 59 d)
[7]First event of at least 3 consecutive days with health score 2 or greater (1 - 59 d)

Table 4. Effect of probiotic on incidence rate of at least one day of a health observation, mortality, removal or farm treatment events in reference to the control group

	Treatment[1]				
	CON		PRO		
Item	n[2]	IRR[3]	n	IRR	95 % CI
Removed (1 - 180 d)[4]	4	1.00	4	1.16	0.30 - 4.57
Mortality (1 - 180 d)	5	1.00	6	1.38	0.43 - 4.43
Fecal score of 3[5]	157	1.00	143	1.04	0.98 - 1.11
Health score of 2[6]	172	1.00	148	0.99	0.96 - 1.01
Health score ≥ 3[7]	53	1.00	52	1.12	0.82 - 1.54
Treated for scours (1 - 28 d)[8]	22	1.00	25	1.28	0.72 - 2.27
Treated for pneumonia					
In hutches (1 - 91 d)	54	1.00	51	1.03	0.75 - 1.41
In pens (92 - 180 d)	94	1.00	86	1.03	0.84 - 1.24

[1]Treatments included a control (CON; Lactose) or Probiotic (PRO; Lactose based *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii*)
[2]n = number of calves affected
[3]IRR = incidence rate ratio of PRO group, CON is reference group
[4]Identified by farm staff as having severe clinical illness and removed from trial
[5]Loose and watery feces observation (1 - 28 d)
[6]Observed dull and fairly responsive demeanor, ears slightly drooped, and eyes slightly sunken at least once (1 - 59 d)
[7]Observed unresponsiveness, drooped ears, difficulty standing, and sunken eyes at least once (1 - 59 d)
[8]No calf reported with scour event after 28 d

Table 5. LSM comparison of fecal pathogen shedding by treatment group

	Treatment				
	CON[1]		PRO[2]		
Item	N[3]	LSM	N	LSM	*P*-Value
Escherichia coli, cfu/g					
7 d	53	3.1×10^8	24	4×10^8	0.3
95% CI		2.5×10^8 - 3.8×10^8		2.3×10^8 - 5.7×10^8	
14 d	53	2.9×10^8	31	2.4×10^8	0.8
95% CI		1.6×10^8 - 5.2×10^8		1.2×10^8 - 4.9×10^8	
21 d	50	8.6×10^7	37	7.1×10^7	0.8
95% CI		4.1×10^7 - 1.8×10^8		3.7×10^7 - 1.4×10^8	
42 d	52	1.6×10^7	37	1.4×10^7	0.9
95% CI		7.4×10^6 - 3.6×10^7		7×10^6 - 3.2×10^7	
Escherichia coli (STEC), cfu/g[4]					
7 d	53	2.9×10^6	20	5×10^6	0.3
95% CI		1.5×10^6 - 5.4×10^6		2.5×10^6 - 1×10^7	
14 d	55	4.2×10^6	28	2.8×10^6	0.3
95% CI		2.7×10^6 - 6.4×10^6		1.7×10^6 - 4.5×10^6	
21 d	52	3.7×10^6	38	3.4×10^6	0.9
95% CI		2×10^6 - 6.8×10^6		2×10^6 - 5.8×10^6	
42 d	51	1.5×10^6	32	1.4×10^6	0.9
95% CI		8.6×10^5 - 2.7×10^6		7.3×10^5 - 2.3×10^6	
Clostridium, cfu/g					
7 d	36	2.7×10^6	10	9.7×10^5	0.3
95% CI		9.1×10^5 - 8.1×10^6		3.5×10^5 - 2.7×10^6	
14 d	42	1.6×10^6	32	1.7×10^6	0.9
95% CI		4.8×10^5 - 5.4×10^6		5.7×10^5 - 5.1×10^6	
21 d	49	2.9×10^5	39	5.4×10^5	<0.05
95% CI		2.1×10^5 - 3.9×10^5		3.6×10^5 - 8.3×10^5	
42 d	43	1.9×10^5	19	1.2×10^7	<0.05
95% CI		1.3×10^5 - 2.6×10^5		4×10^6 - 3.6×10^7	

[1] CON was control treatment (Lactose)

[2] PRO was probiotic treatment (*B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii*)

[3]N = number of calves included in the model

[4]Shinga toxin-producing Escherichia coli

Table 6. Effect of probiotic on incidence rate of shedding *Cryptosporidium* oocyst

	Treatment[1]				
	CON		PRO		
Item	n[2]	IRR[3]	n[2]	IRR[3]	95 % CI
7 d	15	1.00	21	0.97	0.55 - 1.72
14 d	24	1.00	26	1.15	0.76 - 1.73
21 d	6	1.00	12	2.00	0.81 - 4.92
42 d	15	1.00	9	0.47	0.21 - 1.05

[1]Treatments included a control (CON; Lactose) or Probiotic (PRO; Lactose based *B. subtilis*, *B. lichenformis*, *L. animalis*, and *P. freudenreichii*)

[2]n = number of calves affected

[3]IRR = incidence rate ratio of PRO group, CON is reference group

Figure 1. Kaplan-Meier survival of days to first diarrhea.
Reference line indicates median days at first diarrhea for both PRO and CON