Effects of mannan-oligosaccharide and *Bacillus subtilis* supplementation to preweaning Holstein dairy heifers on body weight gain, diarrhea, and shedding of fecal pathogens

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ABSTRACT

The objective of this clinical trial was to evaluate the effectiveness of probiotic, prebiotic, and synbiotic supplementation on average daily weight gain (ADG), duration of diarrhea, age at incidence of diarrhea, fecal shedding of *Cryptosporidium* oocysts, enteric pathogens, and the odds of pneumonia in preweaning dairy heifer calves on a commercial dairy. Feeding prebiotics and probiotics may improve health and production of calves. Hence, healthy Holstein heifer calves ($n = 1,801$) from a large California dairy were enrolled at 4 to 12 h of age and remained in this study until weaning at 60 d of age. Calves were block randomized to 1 of 4 treatments: (1) control, (2) yeast culture enriched with mannan-oligosaccharide (prebiotic), (3) *Bacillus subtilis* (probiotic), and (4) combination of both products (synbiotic), which were fed in milk twice daily from enrollment until weaning. Serum total protein at enrollment and body weight at 7, 42, and 56 d of age were measured. Fecal consistency was assessed daily for the entire preweaning period. A subgroup of 200 calves had fecal samples collected at 7, 14, 21, and 42 d for microbial culture and enumeration of *Cryptosporidium* oocysts by direct fluorescent antibody staining. Synbiotic-treated calves had 19 g increased ADG compared with control calves for overall ADG, from 7 to 56 d. From 42 to 56 d, prebiotic-treated calves had 85 g greater ADG and synbiotic-treated calves had 78 g greater ADG than control calves. There was no difference in duration of the first diarrhea episode, hazard of diarrhea, or odds of pneumonia per calf with treatment. Probiotic-treated calves had 100 times lower fecal shedding of *Cryptosporidium* oocysts at 14 d and

prebiotic-treated calves had fewer *Escherichia coli* and pathogenic *E. coli* at 42 d compared with control calves. Although there were no effects on duration of diarrhea or pneumonia incidence, greater ADG in the late preweaning period may reflect treatment effects on enteric pathogens during the rearing process. The decreased shedding of *Cryptosporidium* should reduce infectious pressure, environmental contamination, and public health risks from *Cryptosporidium*. Our findings suggest ADG and potential health benefits for calves fed prebiotics, probiotics, and synbiotics and can help the dairy industry make informed decisions on the use of these products in dairy production.

Key words: microbial supplement, cryptosporidiosis, mannan-oligosaccharide, *Bacillus subtilis*, dairy calves

INTRODUCTION

Digestive disorders including diarrhea are the most common diseases of preweaning dairy heifers, affecting 25.3% (USDA, 2012). Commonly caused by viral and parasitic agents including *Cryptosporidium parvum* (Bartels et al., 2010), diarrhea is treated with antibiotics in 71.8% of cases (USDA, 2012). The World Health Organization has recommended the need for antibiotic stewardship at a global level (Tacconelli et al., 2018) and the Food and Drug Administration restricts in-feed antibiotic use. Probiotics and prebiotics may improve gut immunity, produce local antimicrobials, decrease pathogen load, and colonize the gastrointestinal tract to prevent colonization (Frizzo et al., 2011; Bajagai et al., 2016). Paired with improved weight gain, this can decrease disease. Probiotics in milk replacer reduce diarrhea compared with in-feed antibiotics (Kim et al., 2011) and offer an alternative to antibiotics.

Currently, 41% of preweaning dairy heifer operations in the United States use probiotics (USDA, 2012) and studies on their effectiveness show mixed results. A

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meta-analysis showed that feeding lactic acid producing bacteria improved feed efficiency after 45 d of age (Frizzo et al., 2011). Studies show increased ADG with a prebiotics or probiotics, as well as decreased diarrhea, shedding of *Escherichia coli*, and reduced clinical severity of *Salmonella* (Timmerman et al., 2005; Roodposhti and Dabiri, 2012; Agazzi et al., 2014; Broadway et al., 2020). Length of diarrhea episode was decreased by almost 1 d when treated with a multispecies probiotic (Renaud et al., 2019). Other studies show no change in feed efficiency or diarrhea (Frizzo et al., 2010). Products of yeast fermentation such as yeast culture, cell walls, refined functional carbohydrates, and mannan-oligosaccharide (MOS) are used as prebiotics. These products increase ADG, hip height, and rumen development, and reduce the number of diarrhea events and treatments (Lesmeister et al., 2004; Galvao et al., 2005; Kara et al., 2015; Melendez et al., 2018). Mannan-oligosaccharide may act to inhibit bacterial and *Cryptosporidium* attachment to the intestinal wall (Chen and LaRusso, 2000; Spring et al., 2000).

Bacillus subtilis alters the rumen microbiome, improves digestion at weaning (Ushakova et al., 2013), and decreases the severity of diarrhea (Kowalski et al., 2009). Feeding *B. subtilis* to Holstein calves increased ADG and levels of serum IgG at weaning, suggesting enhanced immune function, and may decrease incidence of diarrhea, with no previous effect seen on pneumonia (Sun et al., 2010; Melendez et al., 2018). Synbiotics, the use of both probiotics and prebiotics, may support gut health more than independent use, but this has not been extensively studied in ruminants. Clinical disease has been reduced when synbiotics were added to milk (Uyeno et al., 2015; Marcondes et al., 2016). These observations suggest the potential of dietary interventions based on probiotics and prebiotics to increase resistance to pathogens that cause disease.

Cryptosporidium is the leading cause of calf diarrhea, with 50% testing positive between 7 and 21 d (Garber et al., 1994). *Cryptosporidium parvum* is a public health concern as it is present in cattle and associated with human disease (Hunter and Thompson, 2005). A field study in California on 134 Holstein calves supplied probiotics for the first 10 d of life and found no difference in the incidence of diarrhea or shedding of *Cryptosporidium* oocysts (Harp et al., 1996). A study on calves with MOS supplementation in milk replacer found a lower probability of presence of *Cryptosporidium* oocysts in feces for the first 3 wk of the study, but had an insufficient number of calves to measure the direct effect of MOS on *Cryptosporidium* prevalence (Terré et al., 2007). Consequently, there is a need for further studies on these interventions.

The objective of this study was to examine the effects of yeast-derived prebiotics and several strains of *B. subtilis*, both singly and in combination, on ADG, age at first incidence of diarrhea, length of first diarrhea episode, odds of pneumonia, and fecal shedding of enteric bacteria and *Cryptosporidium* oocysts. Our goal was to evaluate the effects on health and performance of Holstein replacement heifers in a commercial setting, thereby providing strong external and internal validity of the study. We hypothesized that the study interventions would improve ADG, reduce the morbidity of diarrhea, and reduce shedding of fecal pathogens.

MATERIALS AND METHODS

Study Design and Population

All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee (protocol number 20291). We conducted a block-randomized, clinical trial. Heifer calves (1,801) were enrolled from a single dairy between January 5 and July 2, 2018. The dairy was an 8,000-milking-cow Holstein herd in Fresno County, California, which raises their own heifers on site. Calves were visually examined by researchers at enrollment and were excluded on presentation with congenital abnormalities or illness. These exclusion criteria included contracted tendons, acidosis, and dehydration. Eleven calves died within 48 h of enrollment and were excluded. All groups were fed the same diet of pasteurized hospital milk enriched with milk balancer and ad libitum starter grain as per

Table 1. Nutrient and chemical composition (% of DM unless noted) of the calf starter and milk balancer

Item	Mean	SD
Calf starter ¹		
DM, g/kg	86.8	1.87
CP	19.25	1.45
Crude fat	3.17	0.25
ADF	10.45	1.10
NDF	17.38	1.10
Lignin	4.08	0.55
Total sugar	6.03	0.46
Ca	1.59	0.59
P	0.84	0.12
Na	0.29	0.03
K	0.88	0.06
Milk balancer		
Protein	4.29	0.53
Fat	3.76	0.49
Lactose	5.13	0.23
SNF	10.98	1.22
MUN	6.00	2.86

¹Ingredients of calf starter: rolled corn, canola pellets, rolled barley, molasses, soybean meal, cottonseed hulls.

Table 2. Comparison of BW, serum total protein, daily starter intake, mortality, and removal rate for treated groups of calves¹

Item	n ²	Treatment ³			
		Control	PRE	PRO	SYN
Birth BW (7 d), kg	1,758	41.9 ± 0.24	41.6 ± 0.24	41.7 ± 0.24	41.9 ± 0.24
Second BW (42 d), kg	1,618	71.3 ± 0.38 ^a	70.7 ± 0.38 ^{ab}	69.9 ± 0.38 ^b	71.2 ± 0.38 ^{ab}
Final BW (56 d), kg	1,602	80.9 ± 0.41 ^{ab}	81.5 ± 0.41 ^a	80.3 ± 0.41 ^b	81.9 ± 0.42 ^a
Total protein, g/dL	1,788	5.60 ± 0.03	5.60 ± 0.03	5.56 ± 0.03	5.56 ± 0.03
Mortality incidence rate (60 d), %	1,797	5.4	6.6	7.8	5.9
95% CI, %		3.6–8	4.6–9.4	5.6–11	4.1–8.7
Removal incidence rate (60 d), %	1,797	2.6	4.2	4.2	5.1
95% CI, %		1.5–4.6	2.7–6.5	2.7–6.6	3.4–7.7

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Each value is ±SE.

²n = number of calves included in model.

³PRE = 14 mL of yeast culture + mannan-oligosaccharide; PRO = 1 g of *Bacillus subtilis*; SYN = combination of PRO and PRE. Reported as marginal LSM.

farm protocol, with starter grain (analyzed by Cumberland Valley Analytical Services) and milk samples taken weekly for nutritional analysis (Tulare Co. DHIA; Table 1).

Sample Size

A sample size of 600 calves per treatment group was estimated to measure a reduction in diarrhea morbidity by 30%, with a power of 0.75 and an α of 0.05. Within the financial and time constraints of this trial, a total of 450 calves were enrolled to the control, prebiotic, and synbiotic groups, and 451 calves to the probiotic group.

Calf Management and Housing

Calves received colostrum within 4 h of birth in the maternity pen and then were moved to calf hutches within 12 h of life. Calves were enrolled twice daily at 0500 and 1700 h and before their first milk feeding. The calf hutches were metal with Tenderfoot flooring (Tandem Products Inc., Minneapolis, MN) raised over flush lanes and contained in 6 barns of 2 rows with high roofs, open sides, and a feeding alley in the middle. Shades could be attached to the sides of the barns to reduce sun and wind exposure. Hutches were connected in rows of 96 with nose to nose contact between neighboring calves and 192 hutches per barn. Milk was a combination of pasteurized transition cow milk from the dairy hospital pen, excluding antibiotic-treated milk, combined with milk replacer powder (Calvamilco Summit High Gain 26:18, Esmilco Inc., Modesto, CA) and milk balancer (Milk Balancer 26:5 P, Esmilco Inc.) to achieve the required volume. Calves were fed 2 L twice daily at 0530 and 1730 h for the first 2 wk. Milk volume was then increased to 6 L/d until weaning at

60 d. At 60 d, milk was reduced to one feeding per day for a week and then milk was completely withdrawn. Calf starter grain and fresh water were always available from birth.

Mortality and Removals

A total of 119 calves died and 75 were removed to the hospital (Table 2). Mortalities were recorded before administration of treatments and dead calves were stored in an onsite freezer and transported once per week to the California Animal Health and Food Safety Laboratory (Tulare, CA) for necropsy. Calves that were determined by farm staff to have severe clinical illness and requiring intensive management were removed from the hutches and transported to a hospital pen. These calves were removed from the trial upon exiting the hutch. Euthanasia decisions were made by the dairy's herd veterinarian upon examination of calves with poor prognosis.

Treatment

Calves were block-randomized at enrollment so that all 4 treatment groups were represented with every 4 enrolled calves. Using Microsoft Excel (2016, Microsoft Corp., Redmond, WA), every possible ordering of the numbers 1 to 4 was generated and replicated to make 450 blocks. A random number list was generated and matched to the first number of each block, then sorted so that the blocks were randomized. The front rail of each calf hutch was painted using a greasepaint stick to indicate the group allocation, by color. The control group (**CON**) received no supplement. The other 3 treatment groups had their treatment added to the milk buckets in front of each hutch after the buckets had

been washed and immediately before milk delivery by the calf feeding staff. Treatments were applied at every milk feeding, twice daily, from the first milk feeding until weaning. Prebiotic calves (**PRE**) were supplemented with 7 mL of a yeast extract (Celmanax, Church and Dwight, Ewing Township, NJ). Probiotic calves (**PRO**) were supplemented with 0.5 g of a commercial *B. subtilis* and *Lactobacillus plantarum* (Certillus, Church and Dwight) equating to 1,000,000,000 and 250,000,000 cfu/head per day, respectively. Synbiotic calves (**SYN**) were supplemented with both treatments at the same rate as in the probiotic and prebiotic treatments.

Data Collection

The trial period began at enrollment and ended at weaning. All measurement data up until a calf finished the preweaning period, died, or was removed was included in analyses. At enrollment, the calf ID, date, time of day (morning or evening), hutch row number, and barn number were recorded. Blood samples were collected from all calves between 24 and 48 h of age by jugular venipuncture using 3-mL red-top serum tubes (BD Vacutainer, Franklin Lakes, NJ). Samples were centrifuged 2 to 3 h after collection for 15 min and measured for serum total protein (g/dL) using a handheld optical refractometer (Protein/Specific Gravity Refractometer, LW Scientific, Lawrenceville, GA) immediately postcentrifugation, or within a maximum of 48 h on refrigerated serum samples.

Fecal scoring was performed once daily after the morning feeding on every calf. Fecal consistency was measured on observation of the freshest feces visible on hutch flooring or in the flush lane under the hutch from behind the calf hutches. A fecal score scale of 1 to 3 was used and adapted from the University of Wisconsin's calf health scoring chart. A score of 1 was normal and formed feces, 2 as semi-formed or loose, and 3 as watery feces (Feldmann et al., 2019). Onset of diarrhea was the age in days at the first recording of a diarrhea score of 3. The end of a diarrhea episode was selected as the second day of 3 consecutive days with a score of less than 3. Length of the first diarrhea episode was calculated as the difference in days between the onset and end of the diarrhea episode.

Calf weighing was performed once weekly using a suspension scale attached to a hydraulic arm. Every calf was weighed 3 times at 7, 42, and 56 d of age (± 6 d). Early ADG (**EDG**) was calculated by dividing the difference in BW from first to second weighing (7–42 d) and dividing by number of days. Late ADG (**LDG**) was calculated by dividing the difference in BW from the second to third weighing (42–56 d) and

dividing by the number of days. Overall ADG (**ODG**) was calculated by dividing the difference in BW from the first to third weighing (7–56 d) and dividing by the number of days.

Fecal sampling was performed once weekly with at least 5 g of feces collected from each calf by digital stimulation and immediately packed on ice. Fifteen percent of all calves were enrolled at birth to a fecal sampling subgroup in groups of 20, once weekly, beginning at the start of the trial. The 20 most recently born calves were enrolled upon arrival at the calf unit on the single day of fecal sampling, which was on the same day of every week. Differences in final numbers of subgroup calves in all analyses are accounted for by calf death or removal, inadequate feces for laboratory analysis or sample damage during the analysis procedure. Fifteen groups of 20 calves, 300 calves total, had fecal samples collected for culture and microbial enumeration. The number of samples obtained was limited by the capacity of the laboratory to rapidly process these samples, which were shipped on the same day in cooler boxes to an external laboratory (Church & Dwight, Waukesha, WI) for processing. Fecal bacteria were enumerated on tryptose sulfite cycloserine agar (Oxoid, CM0587, Basingstoke, UK) with D-cycloserine (400 mg/L) and CHROMagar ECC (CHROMagar, EF322, Springfield, NJ) for the quantification of *Clostridia* and *Escherichia coli*, respectively. Up to 5 representative isolates were harvested from each medium from each fecal sample. The DNA was extracted from pelleted cells of each isolate. Bacterial cells were lysed by incubation for 1 h at 37°C in 200 μ L lysozyme solution (10 mg/mL lysozyme in T₅₀E₁₀) before DNA purification with the Maxwell HT Viral TNA Kit (AX2340, Promega, Madison, WI) according to the manufacturer's methods. *Clostridia* isolates were screened using PCR for the α toxin gene specific to *C. perfringens* (Yoo et al., 1997). *Escherichia coli* isolates were tested using PCR for 9 different virulence genes associated with pathogenic *E. coli*. The first PCR tested for genes *stx1*, *stx2*, *eae*, and *ehxA* (Bai et al., 2012), a method optimized using 4 gene targets, the volume of removed primers was substituted with water. The second PCR tested for genes *iroN*, *ompT*, *hlyF*, *iss*, and *iutA* (Johnson et al., 2008). If an *E. coli* isolate had at least one virulence gene it was considered pathogenic *E. coli*. *Clostridium perfringens* and pathogenic *E. coli* levels were calculated by multiplying the ratio of the target population by the total plate count. Eleven groups of calves from the same fecal sampling subgroup, 220 calves total, were enrolled for *Cryptosporidium* oocyst enumeration beginning at 7 wk of the trial (February 13, 2018) with a 3-wk gap between enrollments.

Quantity of *Cryptosporidium* oocysts were determined by an immunofluorescence antibody technique (Waterborne, New Orleans, LA). Feces were suspended in an equal volume of PBS, centrifuged, and homogenized. Ten microliters of fecal suspension were applied to pre-treated glass slides and labeled with immunofluorescent anti-*Cryptosporidium* antibodies. Slides were viewed using a fluorescence microscope and the total number of oocysts were counted per 10 μ L, and back calculated to estimate oocysts per gram of feces (Pereira et al., 1999; Kilonzo et al., 2013). Feces collected from newborn calves within 12 h of birth were tested for absence of *Cryptosporidium*, then spiked with a known solution of *Cryptosporidium* oocysts to validate fecal processing oocyst recovery.

Diagnosis and treatment of sick calves was carried out as per farm protocol by farm staff. Calves were evaluated and treated twice daily by farm staff for signs of clinical disease. Diarrhea was treated with ampicillin, intravenous fluids, bismuth subsalicylate, and flunixin. Pneumonia was treated with either tulathromycin or enrofloxacin, flunixin, and intravenous fluids as indicated. Treatment records for all antibiotics administered and diagnosis of health conditions were extracted from the farm record database (DHI-Plus, Amelcor, Provo, UT). Pneumonia outcome was assessed as a calf having any record of treatment for pneumonia during the preweaning period as per the farm treatment record.

Statistical Methods

The study unit of interest was the individual calf. Data analyses were performed using Stata 16.0 (College Station, TX). Statistical differences were considered at $P < 0.05$. Data were assessed visually, and one outlier, more than 3 SD higher than the rest of the data, in the variable *Cryptosporidium* counts was removed from the control group. Baseline characteristics at enrollment were compared using one-way ANOVA for BW (kg) and total protein (g/dL). Mortality and removal rates were calculated as incidence rates per 60 d. The outcomes ADG, number of days of first diarrhea, fecal shedding of *Cryptosporidium* oocysts, *E. coli*, pathogenic *E. coli*, *Clostridium*, and *C. perfringens*, and occurrence of pneumonia in calves were evaluated using regression models.

For the outcome ADG, linear regression models were constructed after assessing its normality. Separate models were specified for each of the 3 weight intervals (EDG, LDG, and ODG) as follows:

$$Y_i = \beta_0 + \sum_{a=1}^3 \beta_a T + \beta_4 P + \beta_5 BW + \beta_6 TP + \beta_7 B + e,$$

where Y is the outcome variable, β_0 is the intercept; and $\beta_1 T_1$, $\beta_2 T_2$, and $\beta_3 T_3$ are the fixed effects for treatment (PRE, PRO, and SYN), respectively; $\beta_4 P$ = fixed effect of binary pneumonia occurrence; $\beta_5 BW$ = continuous fixed effect of birth weight (kg); $\beta_6 TP$ = serum total protein above or below 5.5 g/dL; $\beta_7 B$ = fixed effect of block; and e is the residual error.

The outcomes for difference in number of days of first diarrhea event and fecal counts of *Cryptosporidium* oocysts, *E. coli*, pathogenic *E. coli*, *Clostridia*, and *C. perfringens* were not normally distributed and were over-dispersed when evaluated using Poisson regression. Consequently, negative binomial regression models were used to assess these outcomes. The model for each dependent variable (Z) of count data was as follows:

$$\log Z = \beta_0 + \sum_{a=1}^3 \beta_a T + \beta_4 TP + \beta_5 B,$$

where Z were the mean counts; β_0 was the intercept; $\beta_1 T_1$, $\beta_2 T_2$, and $\beta_3 T_3$ are the fixed effects for treatment (PRE, PRO, and SYN), respectively; $\beta_4 TP$ is the serum total protein above or below 5.5 g/dL; and $\beta_5 B$ = fixed effect of block.

The effect of study treatments on the occurrence of pneumonia in calves was evaluated using logistic regression as follows:

$$\text{logit}(P_i) = \beta_0 + \sum_{a=1}^3 \beta_a T_i + \beta_4 BW_i,$$

where P_i was the probability of pneumonia in the i th calf; β_0 was the intercept; and $\beta_1 T_1$, $\beta_2 T_2$, and $\beta_3 T_3$ were the fixed effects for treatment (PRE, PRO, and SYN, respectively); and $\beta_4 BW$ = continuous fixed effect of birth weight (kg).

For each outcome, after evaluating univariate models, a full model was subjected to a manual backward elimination process. The final model was arrived at considering biological importance, statistical significance, and confounder assessment using the method of change in treatment effect estimates by observing a 10% or greater change, and model fit assessed using the Akaike information criteria. In addition, all 2-way interactions were investigated using significance testing (Aly et al., 2010). Final models were validated by assessing the normality of residuals with exception of the logistic regression model for pneumonia, which was assessed using the Hosmer-Lemeshow test for goodness of fit.

Finally, in addition to the negative binomial model for count of days to diarrhea, a Kaplan-Meier analysis

was used to compare the treatment groups' median days to first diarrhea episode, and a Cox proportional hazards (PH) regression model specified with variable selection as described above:

$$h(t) = h_0 t \times \exp\left(\sum \beta_{a=1}^3 \beta_a T_j\right),$$

where t represents survival time, $h_0(t)$ was the hazard function, and the fixed effects β_1 , β_2 , and β_3 were the fixed effects T for treatment variable j (PRE, PRO, and SYN, respectively).

The PH assumption that the hazards of any 2 calves acquiring diarrhea were independent of time (and hence proportional) was assessed using the Schoenfeld test (Klein, 2012). Briefly, scaled Schoenfeld residuals for covariates of noncensored animals were regressed over time, and a significant nonzero slope coefficient was used to identify variables that violated the PH assumption. Violation of the PH assumption was addressed by estimating hazard ratios using an extended Cox model with time-dependent covariates.

RESULTS AND DISCUSSION

Baseline Comparison

The mean BW at first weighing and serum total proteins were 41 kg and 5.6 g/dL and were not different across the treatment groups ($P = 0.73$, $P = 0.41$; Table 2), demonstrating successful randomization in assembling comparable trial groups. There was no difference in incidence rate per 60 d of mortality or removal between treatment groups (Table 2).

Average Daily Gain

Management of the calf in the preweaning period is vital to the production of replacement heifers for the dairy herd. However, due to the calf's immature immunologic state, the preweaning period is a time of high stress and pathogen challenge, morbidity, and mortality. This trial demonstrated the beneficial effect of using a prebiotic or synbiotic supplementation on ADG. All calves that had a recorded weight were included in the ADG outcome analyses. A total of 1,616 calves were included in the linear regression for EDG (7–42 d). Calves in the PRO treatment group gained 27 g/d less than the control group. In this model, calves who experienced an episode of pneumonia gained 33 g/d less than calves who did not. A total of 1,602 calves

were included in the linear regression for LDG. Calves in treatment group PRE or SYN gained 85 and 78 g/d more, respectively, than calves in the control group. Calves with serum total protein greater than 5.5 g/dL gained 48 g/d less than calves with serum total protein lower than 5.5 g/dL. The ODG linear regression model included 1,600 calves (Table 3). Calves in the PRE and SYN group gained 16 and 19 g/d more than the control group in the ODG model. Calves in this model that experienced pneumonia gained 34 g/d less and gained 4 g/d less for every 1 kg increase in initial BW. Calves with serum total protein greater than 5.5 g/dL gained 20 g/d less for the ODG model (Table 2).

A proxy for assessing colostrum delivery and failure of passive transfer is serum total protein at 24 h. When this value was greater than 5.5 mg/dL, the ADG of calves was reduced for ODG and LDG outcomes. The important role of adequate colostrum absorption on the health and growth of the dairy heifer is well documented (Robison et al., 1988; DeNise et al., 1989) and higher serum total protein levels would be expected to enhance growth rates. The mean serum total protein level for the entire cohort of calves was high at 5.6 g/dL with low variation (SD 0.54). A serum total protein measurement of 5.2 g/dL is equivalent to 1,000 mg/dL of serum IgG (Tyler et al., 1996) and considered adequate passive transfer. Less than 30% of the calves in this trial had serum total protein less than 5.2 g/dL and are therefore considered to have a failure of passive transfer. It is possible the calves with lower serum total protein levels experienced a higher rate of ADG later in the preweaning period in compensation for depressed growth earlier, given their poorer immune status and reflecting a good diet and environmental conditions in which these calves were raised.

The effect of treatment on weight gain was more pronounced for the latter half of the preweaning period. In the 2-wk period between 42 and 56 d, calves that received PRE or SYN gained more weight, with an ADG of 80 g/d more than the control group (Table 3). However, this beneficial effect on ADG was not seen in the early portion of the preweaning period (Table 3). Having only 3 weight measurements per calf restricted the estimation of weight gain to 2 periods of the preweaning period. Conventionally milk-fed calves may lose or have very low BW gain in the first week of life (Jasper and Weary, 2002).

No improvement in ADG was seen in the PRO group and the EDG PRO group had an ADG that was 27 g/d less than the control group. This finding differs from other studies that reported increased ADG gain in calves supplemented with *B. subtilis* spp. (Kowalski et

Table 3. Daily BW gain providing LSM of treatments and model coefficients¹

Item	n ²	Treatment ³ intercept				Model coefficient ⁴		
		Control	PRE	PRO	SYN	Pneumonia	Birth BW, kg	Total protein, 5.5 g/dL
Overall BW gain (7–56 d), g/d	1,598	907 ± 31 ^{bc}	923 ± 31 ^{ab}	898 ± 31 ^c	926 ± 31 ^a	–34 ± 9	–4 ± 1	–20 ± 7
Early BW gain (7–42 d), g/d	1,618	696 ± 12 ^a	691 ± 12 ^{ab}	669 ± 12 ^b	698 ± 12 ^a	–33 ± 11	—	—
Late BW gain (42–56 d), g/d	1,600	710 ± 35 ^b	795 ± 35 ^a	760 ± 35 ^{ab}	788 ± 35 ^a	—	—	–48 ± 23

^{a–c}Means within a row with different superscripts differ ($P < 0.05$).

¹Each value is ± SE.

²n = number of calves included in model.

³PRE = 14 mL of yeast culture + mannan-oligosaccharide; PRO = 1 g of *Bacillus subtilis*; SYN = combination of PRE and PRO. Reported as marginal LSM predictions.

⁴All listed coefficients at $P < 0.05$.

al., 2009; Sun et al., 2011). In a meta-analysis on BW gain and feed efficiency in probiotic-supplemented pre-weaning dairy calves an increase in BW gain was found (Frizzo et al., 2011). When partitioned by feed type, this effect was only seen in milk replacer-fed calves. In our trial, milk feeding was a combination of pasteurized whole milk sourced on farm, supplemented with commercial milk replacer and balancer, and we measured ADG instead of BW gain. *Bacillus* spp. are considered normal organisms in the soil, and since whole milk was used, there was potential for environmental or fecal contamination with *B. subtilis*. Whole milk is assumed to have a greater level of bacterial flora than milk replacer feeds, which may diminish the potential effect on growth performance of probiotic supplementation.

Lesmeister et al. (2004) found increased rumen papillae length and width in weaned Holstein calves that were supplemented with yeast culture products in the starter grain. The addition of PRE to the milk fed in this trial may have enhanced rumen development and the calf's feed conversion of starter grain. These calves exhibited a superior weight gain in the latter half of the preweaning period when grain intake is an important dietary component. Our improved ADG finding for PRE is consistent with previous literature (Galvao et al., 2005; Ghosh and Mehla, 2012), and identified increased ADG in the PRE and SYN treatments in Holstein heifers on a commercial calf rearing facility.

Diarrhea Analysis

The negative binomial regression was used to assess the difference between the number of days of the first diarrhea event for each calf. In total, 1,693 calves ex-

perienced diarrhea and were included in the model. No difference was observed between the treatment groups (Table 4).

A total of 1,797 calves were included in the Kaplan-Meier survival analysis of time to first diarrhea. Four calves died between enrollment and their first fecal score and were censored. In total, 1,693 calves experienced diarrhea and were included as failure events. The median time to diarrhea was 8 d and did not differ among groups (Figure 1). A Cox proportional hazard regression model for first diarrhea hazard was conducted with no difference in hazard of diarrhea between treatment groups (Table 5).

We chose to examine the age at and duration of the first diarrhea event, as this event can influence performance in the preweaning period, given the calves' young age and immature immune system. The mean age at first diarrhea was 8.7 d, with no difference across treatment groups.

Cryptosporidium and Bacterial Fecal Shedding

A total of 156, 160, and 195 calves were included in regression analysis of *Cryptosporidium* oocyst shedding for 7, 14, and 21 d, respectively. Three groups of 20 calves were excluded from 7 and 14 d sampling and one group was excluded from the 21-d sample due to handling error. Other exclusion criteria were sample loss or damage and death of calves before the sample age. The prevalence of oocyst shedding was 87, 89, and 34% with a mean of 259,250, 230,000, and 915 oocysts/g at 7, 14, and 21 d, respectively. No difference in quantity of oocysts per g of feces by group was shown at 7 or 21 d. Calves in the PRO group had a 10-fold lower number

Table 4. Negative binomial regression for length of the first diarrhea episode (d) providing LSM of fecal pathogen shedding for treatment

Treatment ²					
Item	n ¹	Control	PRE	PRO	SYN
Diarrhea length, ³ d	1,693	4.38 ± 1.03	4.41 ± 1.03	4.27 ± 1.03	4.36 ± 1.03
Cryptosporidium, oocysts/g					
7 d ⁴	156	277,462	244,543	199,606	318,245
95% CI		138,026–557,757	121,650–491,584	100,195–397,647	156,877–645,596
14 d ⁵	160	1,460,150 ^a	160,924 ^{ab}	12,644 ^b	337,904 ^a
95% CI		163,274–13,058,100	20,873–1,240,630	1,146–139,410	44,182–1,240,630
21 d ⁴	160	785 ^{ab}	2,201 ^a	395 ^{ab}	280 ^b
95% CI		214–2,870	610–7,940	108–1,446	79–995
<i>Escherichia coli</i> , cfu/g					
7 d ⁶	265	7.7 × 10 ⁷ ^{ab}	7.6 × 10 ⁷ ^{ab}	1.03 × 10 ⁸ ^a	4.4 × 10 ⁷ ^b
95% CI		3.8 × 10 ⁷ –1.17 × 10 ⁸	3.7 × 10 ⁷ –1.15 × 10 ⁸	5.1 × 10 ⁷ –1.15 × 10 ⁸	2.3 × 10 ⁷ –6.6 × 10 ⁷
21 d ⁷	243	1.7 × 10 ⁷	2.7 × 10 ⁷	1.8 × 10 ⁷	1.8 × 10 ⁷
95% CI		3.9 × 10 ⁶ –3.1 × 10 ⁷	5.6 × 10 ⁶ –4.8 × 10 ⁷	4.6 × 10 ⁶ –3.2 × 10 ⁷	4.1 × 10 ⁶ –3.1 × 10 ⁷
42 d ⁸	267	1.6 × 10 ⁷ ^a	7.6 × 10 ⁶ ^b	1.3 × 10 ⁷ ^{ab}	1.4 × 10 ⁷ ^{ab}
95% CI		8 × 10 ⁶ –2.4 × 10 ⁷	3.5 × 10 ⁶ –1.1 × 10 ⁷	7 × 10 ⁶ –1.9 × 10 ⁷	7 × 10 ⁶ –2.1 × 10 ⁷
Pathogenic <i>E. coli</i> , cfu/g					
7 d ⁹	259	4.2 × 10 ⁷	4.8 × 10 ⁷	6.3 × 10 ⁷	3.3 × 10 ⁷
95% CI		1.5 × 10 ⁷ –6.7 × 10 ⁷	1.9 × 10 ⁷ –7.6 × 10 ⁷	2.6 × 10 ⁷ –1 × 10 ⁸	1.3 × 10 ⁷ –5.2 × 10 ⁷
21 d ⁴	240	2 × 10 ⁷	2.6 × 10 ⁷	2.6 × 10 ⁷	1.7 × 10 ⁷
95% CI		0–4.1 × 10 ⁷	0–5.3 × 10 ⁷	0–5.3 × 10 ⁷	0–3.5 × 10 ⁷
42 d ⁶	257	1 × 10 ⁷ ^a	3.9 × 10 ⁶ ^b	6 × 10 ⁶ ^{ab}	9 × 10 ⁶ ^{ab}
95% CI		4.2 × 10 ⁶ –1.6 × 10 ⁷	1.6 × 10 ⁶ –6.3 × 10 ⁶	2.7 × 10 ⁶ –9.8 × 10 ⁶	3.6 × 10 ⁶ –1.5 × 10 ⁷
<i>Clostridium</i> spp., cfu/g					
7 d ¹⁰	285	4.4 × 10 ⁶	6.8 × 10 ⁶	5.3 × 10 ⁶	3.9 × 10 ⁶
95% CI		9 × 10 ⁴ –8.7 × 10 ⁶	2.7 × 10 ⁵ –1.3 × 10 ⁷	1 × 10 ⁵ –1 × 10 ⁷	7.3 × 10 ⁴ –7.8 × 10 ⁶
21 d ⁶	258	5.7 × 10 ⁴	2.1 × 10 ⁴	3.9 × 10 ⁴	5.3 × 10 ⁴
95% CI		1 × 10 ⁴ –1 × 10 ⁵	3.9 × 10 ³ –3.8 × 10 ⁴	7.2 × 10 ³ –7 × 10 ⁴	1 × 10 ⁴ –9.6 × 10 ⁴
42 d ⁶	266	1.3 × 10 ⁴	1.4 × 10 ⁴	1.1 × 10 ⁴	1.2 × 10 ⁴
95% CI		0–2.7 × 10 ⁴	0–2.9 × 10 ⁴	0–2.3 × 10 ⁴	0–2.5 × 10 ⁴
<i>C. perfringens</i> , cfu/g					
7 d ⁶	284	3.9 × 10 ⁶	1.2 × 10 ⁷	7 × 10 ⁶	3.6 × 10 ⁶
95% CI		0–1.1 × 10 ⁷	0–3.5 × 10 ⁷	0–2 × 10 ⁷	0–1 × 10 ⁷
21 d ¹¹	255	3.8 × 10 ⁵	2 × 10 ⁵	1.9 × 10 ⁵	4.6 × 10 ⁵
95% CI		0–9.5 × 10 ⁵	0–5.52 × 10 ⁵	0–4.7 × 10 ⁵	0–1 × 10 ⁶
42 d ⁴	263	3.4 × 10 ⁴	5.3 × 10 ⁴	3.2 × 10 ⁴	3.6 × 10 ⁴
95% CI		0–8.6 × 10 ⁴	0–1.3 × 10 ⁵	0–7.9 × 10 ⁴	0–8.8 × 10 ⁴

^{ab}Means within a row with different superscripts differ ($P < 0.05$).¹n = number of calves included in model.²PRE = 14 mL of yeast culture + mannan-oligosaccharide; PRO = 1 g of *Bacillus subtilis*; SYN = combination of PRE and PRO.³Model included total protein above or below 5.5 g/dL as a fixed effect.⁴Model included only treatment as a fixed effect.⁵Model included block and interaction term between block and treatment as fixed effects.⁶Model included month of birth as a fixed effect.⁷Model included days of age at first diarrhea as a fixed effect.⁸Model included month of birth, total protein above or below 5.5 g/dL, and birth weight as fixed effects.⁹Model included month of birth, total protein level above or below 5.5 g/dL, birth weight, and an interaction term between birth weight and total protein above or below 5.5 g/dL as fixed effects.¹⁰Model included month of birth and total protein above or below 5.2 g/dL as fixed effects.¹¹Model included month of birth, BW, and total protein above or below 5.2 g/dL as fixed effects.

Figure 1. Kaplan-Meier survival model of days to first diarrhea. Reference line indicates median survival time.

of oocysts than calves in the control group with 12,644 and 1,460,150 oocysts/g of feces, respectively (Table 4).

Previous studies in calves found no difference in *Cryptosporidium* shedding through probiotic use (Harp et al., 1996). Using a quantitative method of *Cryptosporidium* detection allowed us to explore the shedding of oocysts as a continuous outcome, rather than with semi-qualitative methods available such as the modified Ziehl-Nielsen stain (Casemore et al., 1985). Given the low infectious dose and high prevalence of *Cryptosporidium* incidence in affected herds, a quantitative method gave us more information about individual calf parasite burden. The quantity of oocysts at 7 and 21 d were not different across the treatment groups, and were numerically lower than the number of oocysts/g at 14 d. This is consistent with current knowledge regard-

ing the epidemiology of *Cryptosporidium* infections in dairy calves, as peak shedding prevalence is observed in the second week of life, resulting from infection beginning at 1 wk and resolving at 3 wk (Santin et al., 2004). The effect of PRO was only evident at peak shedding but may reduce the environmental burden of *Cryptosporidium* oocysts present in the calf facility. The mode of action of the prebiotic and the probiotic is expected to be separate and not interacting, with prebiotics binding to bacteria and pathogens and decreasing colonization, and probiotics enhancing the immune response, and producing compounds that kill competing pathogens. The *Bacillus subtilis* in the PRO group is not expected to bind *Cryptosporidium*, but instead inhibit its ability to adhere to and reproduce using enterocytes by modulating the local immune re-

Table 5. Hazard ratio of survival time to first episode of diarrhea

Item	n ¹	Treatment ²			
		Control	PRE	PRO	SYN
Diarrhea, hazard ratio	1,797	Referent	1.01 ± 0.07	0.96 ± 0.07	1.02 ± 0.07

¹n = number of calves included in model.

²PRE = 14 mL of yeast culture + mannan-oligosaccharide; PRO = 1 g of *Bacillus subtilis*; SYN = combination of PRE and PRO.

sponse. The probiotic bacteria may have been bound to the prebiotic when used in the SYN group, which would decrease its local effect, leading to no observed change on *Cryptosporidium* in the SYN group. The calf groups enrolled in *Cryptosporidium* sampling were a subpopulation of the groups enrolled for microbiologic sampling, which were collected from March to May at irregular intervals. Such sampling represented calves across the spring and early summer seasons of this region, which has diverse weather and moisture conditions, with greater precipitation in the spring season of the trial and cooler temperatures when compared with summer. The statistical model developed included a term for enrollment block and an interaction between the treatment and block. These effects may control for a difference in seasonal shedding of *Cryptosporidium*, as higher shedding concentrations were observed in May compared with June, July, and August (Atwill et al., 1999).

The infective dose of *Cryptosporidium* is considered low and can be transmitted from dam to calf at parturition, as adult cows may shed increased quantities of *Cryptosporidium* peri-parturiently and contaminate the maternal environment. Prompt removal of calves from the maternity area reduces the number of clinical *Cryptosporidium* cases (Faubert and Litvinsky, 2000) and this was the practice on the trial farm. It is assumed, therefore, that most of the *Cryptosporidium* exposure during this trial was horizontal within the calf unit. Commercial preweaning dairy calf rearing is commonly conducted in individual hutches, with a strategy of “all in and all out.” Calves enter their hutch after birth and leave postweaning, allowing for cleaning and disinfection of hutches between calves. Hutches are usually managed in lines, or above flush lanes, with variable risk of horizontal contamination. By reducing the overall quantity of *Cryptosporidium* oocysts excreted per calf, the overall infectious pressure and risk of transmission of the disease within the system may decrease. This calf system was managed above flush lanes and *Cryptosporidium* exposure may occur from splashing or contamination from the wastewater under-

neath the hutches, or by fomite spread. Widespread use of the studied probiotic in this system would decrease the quantity of oocysts shed into the environment and decrease the exposure level, potentially lowering the number of calves affected by *Cryptosporidium* diarrhea and consequently decreasing the motivation to use antibiotics. Housing type and management practices should be stated and compared in further studies as differences in exposure and challenge levels, as well as stressors, may modify this effect on *Cryptosporidium* shedding or the clinical expression of diarrhea.

Genetic typing of the oocysts isolated in this trial was not performed, but cattle can shed *C. andersoni*, *C. bovis*, and *C. parvum*. *Cryptosporidium parvum* is considered to have the highest zoonotic potential. High dairy use areas are associated with *Cryptosporidium* contaminated storm run-off, with calf raising associated with increased environmental loading. Beneficial farm management protocols to reduce this risk include buffers and barriers, to trap and impede overland flow of oocysts and remove them from run-off. An overall reduction in calf unit environmental loading of *Cryptosporidium* using probiotic supplementation may be a beneficial practice to improve water quality (Miller et al., 2008).

Between 285 and 261 calves were included in the negative binomial models for shedding of the 4 types of bacterial colonies. The number of calves differed with calf loss due to death, or sampling producing an inadequate quantity of feces. Quantities of *E. coli* and pathogenic *E. coli* species were lower for the PRE group at 42 d (Table 4). This effect was only seen later in the preweaning period. Sustained supplementation of probiotic species may be necessary to affect a change in the microbiota and achieve competitive exclusion effects. The preweaning period is when the supplemented species establishes itself in the gastrointestinal system and can reduce colonization by pathogenic organisms (Callaway et al., 2008). *Escherichia coli* species are a common inhabitant of the bovine gastrointestinal tract and are important food safety pathogens. Interventions that reduce the prevalence of these organisms may be

Table 6. Odds ratio of at least 1 pneumonia event

Item	n ¹	Treatment ²				Model coefficient ³ (birth BW), kg
		Control	PRE	PRO	SYN	
Pneumonia, odds ratio	1,758	Referent	0.82 ± 1.03	0.95 ± 0.17	0.82 ± 0.15	1.03 ± 0.01

¹n = number of calves included in model.

²PRE = 14 mL of yeast culture + mannan-oligosaccharide; PRO = 1 g of *Bacillus subtilis*; SYN = combination of PRE and PRO.

³All listed coefficients at $P < 0.05$.

valuable in controlling the risk of coliform disease as a function of reduced exposure of calves to environmental opportunistic pathogens.

Pneumonia Treatment

The logistic regression model included 1,797 calves to estimate the odds ratio of a calf diagnosed and treated for pneumonia. There was no difference in the odds of being treated for pneumonia across the 4 treatment groups (Table 6). Despite the potential improvement in immune function and increased serum IgG previously reported in *Bacillus subtilis*-supplemented calves, we saw no difference in the clinical presentation and treatment level of pneumonia in this trial, similar to previous work in smaller samples of calves (Melendez et al., 2018).

CONCLUSIONS

This study is one of the first large field trials to evaluate not only probiotic and prebiotic use, but also synbiotic supplementation in dairy calves. Given that yeast products differ in manufacture and formulation and that strains of bacteria may differ in effect on calves, studies with sufficient power to evaluate the performance of these, singly or in combination are useful to provide evidence of efficacy. The results provided here can be used to inform decisions on the use of these products in dairy production. Both PRE and SYN increased ADG over CON, but the PRO treatment showed no effect on weight gain in isolation. Given the greater weight gain, particularly later in the preweaning period, and the potential effects of prebiotic supplementation on rumen development, calf health, and growth rates should be studied beyond the preweaning period. There was reduced shedding of *Cryptosporidia* for the PRO group and reduced fecal presence of pathogenic and nonpathogenic *E. coli* for the PRE group.

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