### Title

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## INTERPRETIVE SUMMARY

**Lucey et al**: Effects of mannan-oligosaccharide and *Bacillus subtilis* supplementation to pre-wean Holstein dairy heifers on bodyweight gain, diarrhea, and shedding of fecal pathogens

Digestive disorders such as diarrhea are the most common diseases of pre-wean dairy heifers and reducing antibiotic use are of vital interest to the dairy industry. We investigated whether feeding a probiotic and prebiotic, singly or in combination could improve calf health or production in a large clinical trial on a commercial dairy. Calves treated with prebiotics and probiotics had increased gain, probiotic calves shed fewer *Cryptosporidium* oocysts at 14 d of age and prebiotic treated calves had reduced fecal presence of pathogenic and non-pathogenic *E. coli* than controls. The results provided here can be used to inform decisions on the use of these products in dairy production.

# Effects of mannan-oligosaccharideand *Bacillus subtilis* supplementation to pre-wean Holstein dairy heifers on bodyweight 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 pre-wean 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 –12 h of age and remained in this study until weaning at 60 d of age. Calves were block-randomized to 1 of 4treatments: 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 pre-wean 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. Synbiotictreated calves had 19 g increased ADG compared to control calves for overall ADG,from 7 to 56 d. From42 to 56 d, prebiotic treated calves had 85 g greater ADG and synbiotictreated calves had 78 g greater ADG than control calves. There was no difference in durationof 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*E. coli* and pathogenic *E. coli* at 42 dcompared to control calves.Although there were no effects on duration of diarrhea or pneumonia incidence, greaterADG in the late pre-wean period may reflect treatment effects on enteric pathogens during the rearing process.The decreased shedding of *Cryptosporidium*shouldreduce 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.

KEYWORDS: Microbial supplement, cryptosporidiosis, mannan-oligosaccharide, Bacillus subtilis, dairy calves

## INTRODUCTION

Digestive disorders including diarrhea, are the most common diseases of pre-wean 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 hasrecommended 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 (Bajagai, 2016; Frizzo et al., 2011).Paired with improved weight gain this can decrease disease. Probiotics in milk-replacerreducediarrhea compared within-feed antibiotics (Kim et al., 2011) and offer an alternative to antibiotics.

Currently, 41% of pre-wean dairy heifer operations in the U.S. use probiotics(USDA, 2012)and studies ontheir effectiveness show mixed results. A meta-analysisshowed that feeding lactic acid producing bacteria improved feed efficiency after 45 d of age(Frizzo et al., 2011).Studies show increased ADGwitha 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 usedas prebiotics. They increaseADG, hip height, rumen development, and reduce the numberof 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

92 *Cryptosporidium* attachment to the intestinal wall (Chen and LaRusso, 2000; Spring et al.,
93 2000).
94
95 *Bacillussubtilis* alters the rumen microbiome, improves digestion at weaning (Ushakova et
96 al., 2013) and decreases the severity of diarrhea (Kowalski et al., 2009). Feeding *B. subtilis* to
97 Holstein calves increased ADG and levels of serum IgG at weaning, suggesting enhanced
98 immune functionand may decrease incidence of diarrhea, with no previous effect seen on
99 pneumonia (Sun et al., 2010;Melendez et al., 2018). Synbiotics, the use of both probiotics and
100 prebiotics, may support gut health more than independent use, butthis has not been
101 extensively studied in ruminants.Clinical diseasehas been reduced when synbiotics
102 wereadded to milk(Uyeno et al., 2015; Marcondes et al., 2016). These observations suggest
103 the potential of dietary interventions based on probiotics and prebiotics to increase resistance
104 to pathogens that cause disease.
105
106 *Cryptosporidium* is the leading cause of calf diarrhea, with 50% testing positivebetween 7
107 and 21 d (Garber et al., 1994).*C. parvum*is a public health concern as it is present in cattle
108 andassociated with human disease(Hunter and Thompson, 2005).A field study in California
109 on 134 Holstein calves suppliedprobiotics for the first 10 d of life and found no difference in
110 the incidence of diarrhea or shedding of *Cryptosporidium*oocysts (Harp et al., 1996). A study
111 on calves withMOS supplementation in milk-replacerfound a lower probability of presence of
112 *Cryptosporidium* oocysts in feces for the first 3 wk of the study, but had an insufficient
113 number of calves to measure the direct effect of MOS on *Cryptosporidium* prevalence (Terré
114 et al., 2007). Consequently, there is a need for further studies on these interventions.
115 The objective of this study was to examine the effects of yeast derived prebioticsand several
116 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 theeffects on health and performance of Holstein replacement heifersin 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 AnimalCare and Use Committee (protocol number #20291). We conducted a block randomized, clinical trial. Heifer calves (1,801) were enrolled from a single dairy between the 5$^{th}$ of January and the 2$^{nd}$ of July 2018. The dairy was an 8,000-milking cow Holstein herd in Fresno County, CAwhichraises their own heiferson 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 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 alpha 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 weremoved to calf hutches within 12 h of life. Calves were enrolled twice daily, at05:00 and 17:00and before their first milk feeding. The calf hutches were metal with Tenderfootflooring (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 connectedin 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,excludingantibiotic treated milk, combined with milk replacer powder (CALFMILCO Summit High Gain 26:18, Esmilco Inc, Modesto, CA) and milk balancer (Milk Balancer 26:5 P, Esmilco Inc., Modesto, CA) to achieve the required volume. Calves were fed 2L twice daily at 05:30 and17:30 for the first 2 wk. Milk volume was then increased to 6L per day 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 wk 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 uponexamination 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), every possible ordering of the numbers 1 – 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 7mL of a yeast extract (CELMANAX™, Church and Dwight, Ewing Township, NJ). Probiotic calves (PRO) were supplemented with 0.5g of a commercial *B. subtilis*and *Lactobacillus plantarum*(CERTILLUS™, Church and Dwight, Ewing Township, NJ) equating to 1,000,000,000 CFU/head/d and 250,000,000 CFU/head/d 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 pre-wean 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 mLred top serum tubes (BD Vacutainer, Franklin Lakes, NJ). Samples were centrifuged 2 - 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, GA)immediately post centrifugation, 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 – 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 wasperformed 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 (± 6d). Early ADG (EDG) was calculated by dividing the difference in bodyweightfromfirst to second weighing (7 – 42 d) and dividing by number of days.Late ADG (LDG) was calculated by dividing the difference in bodyweight 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 bodyweight from the first to third weighing (7 – 56 d) and dividing by the number of days.

Fecal samplingwas performed once weekly with at least5 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 afecal sampling subgroup in groups of twenty,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, whichwere 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 (TSC) agar (Oxoid, CM0587, Hampshire) with D-cycloserine (400 mg/L) and CHROMagar™ ECC (CHROMagar, EF322, New Jersey) for the quantification of *Clostridia* and *Escherichia coli*, respectively. Up to five representative isolates were harvested from each media from each fecal sample. The DNA was extracted from pelleted cells of each isolate. Bacterial cells were lysed by incubation for one hour at 37°C in 200 µl lysozyme solution (10 mg/mL lysozyme in $T_{50}E_{10}$) before DNA purification with the Maxwell® HT Viral TNA Kit (Promega, AX2340, Wisconsin) according to the manufacturer's methods. Clostridia isolates were screened using PCR for the alpha toxin gene specific to *C. perfringens*(Yoo et al., 1997). *E. coli* isolates were tested using PCR for nine 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 four 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*. *C. 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 at7wk of the trial (2/13/2018) with a 3 wk gap between enrollments.

Quantity of *Cryptosporidium*oocysts were determined by animmunofluorescence antibody technique (Waterborne, New Orleans, LA). Feces were suspended in an equal volume of phosphate-buffered saline,centrifuged and homogenized. Ten µL of fecal suspension were applied to pretreated 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 µL, and back calculated to estimate oocysts per g 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, Amelicor, Provo,

UT).Pneumonia outcome was assessed as a calf having any record of treatment for pneumonia during the pre-wean 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 oneoutlier, 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 bodyweights (kg) andtotal protein (g/dL). Mortality and removal rates were calculated as incidence rates per 60 d. The outcomes ADG, number of d 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,mixed effects linear regression models were constructed after assessing its normality. Separate models were specified for each of the3 weight intervals: EDG, LDG, and ODG as follows:

$$Y_i = \beta_0 + \sum_{a=1}^{3} \beta_a T_i + \beta_4 P_i + \beta_5 BW_i + \beta_6 TP_i + \beta_7 B_i + u_{0\,i}^{calf} + e_i$$

Where$\beta_0$was the intercept, and $\beta_1 T_1$, $\beta_2 T_2$, $\beta_3 T_3$were the fixed effects for treatments PRE, PRO and SYN, respectively;$\beta_4 P$ = fixed effect of binary pneumonia occurance; $\beta_5 BW$ =continuous fixed effect of birthweight (kg); $\beta_6 TP$ = serumtotal protein above or below 5.5g/dL; $\beta_7 B$ = fixed effect of block. The random effects for calf and residual errors were assumed to be distributed univariate normal with means 0 and variance $\sigma^2_{calf}$ and $\sigma^2_{residual}$, respectively.

The outcomes for difference in number of daysof 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, mixed effectsnegative binomial regression models were used to assess these outcomes.The model for each dependent variable (*Z*) of count data was as follows:

$$logZ_i = \beta_0 + \sum_{a=1}^{3}\beta_a T_i + \beta_4 TP_i + \beta_5 B_i + u_{0\,i}^{calf}$$

Where$Z_i$were the mean counts in the $i^{th}$ calf,$\beta_0$was the intercept, and $\beta_1 T_1$, $\beta_2 T_2$, $\beta_3 T_3$were the fixed effects for treatments PRE, PRO and SYN, respectively;$\beta_4 TP$ = serumtotal protein above or below 5.5g/dL; $\beta_5 B$ = fixed effect of block. The random effects for calf and residual errors were assumed to be distributed univariate normal with means 0 and variance $\sigma^2_{calf}$ and $\sigma^2_{residual}$, respectively.

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

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

Where$P_i$ was the probability of pneumonia in the $i^{th}$calf,$\beta_0$was the intercept, and $\beta_1 T_1$, $\beta_2 T_2$, $\beta_3 T_3$were the fixed effects for treatments PRE, PRO and SYN, respectively;$\beta_4 BW$ = continuous fixed effect of birthweight (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, 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 (AIC). In addition, all two-way interactions were

311 investigated using significance testing (Aly et al., 2010). Final models were validated by
312 assessing the normality of residuals with exception of the logistic regression model for
313 pneumonia which was assessed using the Hosmer-Lemeshow test for goodness of fit.
314
315 Finally, in addition to the negative binomial model for count of days to diarrhea, a Kaplan-
316 Meier analysis was used to compare the treatment groups' median days to first diarrhea
317 episode, and a Cox Proportional Hazards (PH) regression model specified with variable
318 selection as described above.
319 $$h(t) = h_o t \times \exp(\sum \beta_{a=1}^{3} \beta_a T_i)$$
320 Where *t* represents survival time, *h(t)*was the hazard function and the fixed effects $\beta_1$, $\beta_2$,
321 $\beta_3$were the fixed effects *T* for treatment variable PRE, PRO and SYN, respectively.
322
323 The PH assumption that thehazards of any 2 calvesacquiring diarrheawere independentof
324 time (and hence proportional) was assessedusing the Schoenfeld test(Klein, 2012). Briefly,
325 scaled Schoenfeld residuals for covariates ofnon-censored animals were regressed over time,
326 and asignificant non-zero slope coefficient was used to identifyvariables that violated the PH
327 assumption. Violationof the PH assumption was addressed by estimatinghazard ratios using
328 an extended Cox model with timedependentcovariates.
329
330 **RESULTS AND DISCUSSION**
331 ***Baseline comparison***
332 The mean bodyweight at first weighing and serum total proteinswere 41kg and 5.6g/dLand
333 were not different across the treatment groups ($P$=0.73, $P$=0.41; Table 2)demonstrating
334 successful randomization in assembling comparable trial groups. There was no difference in
335 incidence rate per 60 d of mortality or removalbetween treatment groups (Table 2).

***Average daily gain***

Management of the calf in the pre-wean period is vital to the production of replacement heifers for the dairy herd. However, due to the calf's immature immunologic state, the pre-wean 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 SYNgained 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 serumtotal 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 more per d than the control group in the ODG model. Calves in this model that experienced pneumonia gained 34 g/d less, andgained 4g/d less for every 1 kg increase in initial bodyweight. Calves with serum total protein greater than 5.5g/dL gained 20g/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 than5.5 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/dLwith low variation

(SD 0.54). A serum total protein measurement of 5.2 g/dLis 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, 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 pre-wean 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 pre-wean period. In the 2-week period between 42 d and 56 d, calves that received PREor SYNgainedmore weight, with an ADGof 80 g/d more than the control group (Table 3). However, this beneficial effect onADG was not seen in the early portion of the pre-wean period (Table 3). Having only 3 weight measurements per calf restricted the estimation of weight gain to 2 periods of the pre-wean period.Conventionally milk fed calves may lose or have very low body weight gain in the first week of life (Jasper and Weary, 2002).

No improvement in ADG was seen in the PRO group and theEDGPRO group had an ADG that was 27 g/d less than the control group. This finding differs from other studies that reported increased ADGgain in calves supplemented with *B. subtilis spp.* (Kowalski et al., 2009; Sun et al., 2011). In a meta-analysis onbody weight gain and feed efficiencyin probiotic supplemented pre-wean dairy calves an increase in bodyweight 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 body weight 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 pre-wean 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 inthe 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 experienced 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 thisevent caninfluenceperformance in the pre-wean 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 *Cryptosporidum*oocyst shedding for 7, 14, and 21 d, respectively. Three groups of 20 calves were excluded from 7 d and 14 d sampling and one group was excluded from the 21 d sampledue to handling error. Other exclusion criteria was sample loss or damage and death of calves prior to the sample age. The prevalence of oocyst shedding was 87, 89, and 34% with a mean of 259,250, 230,000, and 915 oocysts/gat 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 ten fold lower number 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-Nielsenstain (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 regarding the epidemiology of *Cryptosporidium* infections in dairy calves, as peak shedding prevalence is observed in the second week of life, resulting from infection beginning at1wk and resolving at3 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.We expect the mode of action of the prebiotic and the probiotic was separate and would not interact, with prebiotics binding bacteria and pathogens and decreasing colonization, and probiotics enhancing the immune response, and producing compounds that kill competing pathogens. We do not expect that the *Bacillus subtilis* in the PRO group would bind *Cryptosporidium*, but instead inhibit its ability to adhere to and reproduce using enterocytes by modulating the local immune response. The action of the probiotic in the SYN group may have been inhibited by binding to mannan-oligosaccharide, leading to no observed effect on C*ryptosporidium* in the SYN group. The calf groups enrolled in *Cryptosporidium* sampling were a sub-population of the groups enrolled for microbiologic sampling, which were collected from March to May at irregular intervals.Such sampling representedcalves across the Spring and early Summer seasons of this region, which hasdiverse weather and moisture conditions, with greater precipitation in the Spring season of the trial and cooler temperatures when compared to 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 to June, July, and August (Atwill et al., 1999).

The infective dose of *Cryptosporidium* is considered low and can be transmitted from damto calf at parturition, as adult cows may shed increased quantities of *Cryptosporidium* peri-parturientlyand 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 pre-wean 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 post-weaning, 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 underneath 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. C. parvum*is considered to have the highest zoonotic potential. High dairy use areas are associated with *Cryptosporidium* contaminated storm run-

484 off, with calf raising associated with increased environmental loading. Beneficial farm
485 management protocols to reduce this risk include buffers and barriers, to trap and impede
486 overland flow of oocysts and remove them from run-off. An overall reduction in calf unit
487 environmental loading of *Cryptosporidium*using probiotic supplementation may be a
488 beneficial practice to improve water quality (Miller et al., 2008).
489
490 Between 285 and 261 calves were included in the negative binomial models for shedding of
491 the 4 types of bacterial colonies. The number of calves differed with calf loss due to death, or
492 sampling producing an inadequate quantity of feces. Quantities of *E. coli* and pathogenic *E.*
493 *coli* species were lower for the PRE group at 42 d (Table 4).This effect was onlyseen later in
494 the pre-wean period.Sustained supplementation of probiotic species may be necessary to
495 affect a change in the microbiota and achieve competitive exclusion effects.The pre-wean
496 periodis when the supplemented species establishes itself in the gastrointestinal system and
497 can reduce colonization by pathogenic organisms (Callaway et al., 2008). *E. coli* species are a
498 common inhabitant of the bovine gastrointestinal tract and are important food safety
499 pathogens. Interventions that reduce the prevalence of these organisms may be valuable in
500 controlling the risk of coliform disease as a function of reduced exposure of calves to
501 environmental opportunistic pathogens.
502
503 ***Pneumonia treatment***
504 The logistic regression model included 1,797 calves to estimate the odds ratio of a calf
505 diagnosed and treated forpneumonia. There was no difference in the odds of being treated for
506 pneumonia across the 4 treatment groups (Table 6). Despite the potential improvement in
507 immune function and increased serum IgG previously reported in *Bacillus*
508 *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).

## CONCLUSION

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 pre-wean period and the potential effects of prebiotic supplementation on rumen development, calf health and growth rates should be studied beyond the pre-wean period. There was reduced shedding of *Cryptosporidia*for the PRO group and reduced fecal presence of pathogenic and non-pathogenic *E. coli*for the PRE group.

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712

713 **Table 1.** Nutrient and chemical composition of the calf starter and milk balancer

| Item, % of DM | Mean | SD |
|---|---|---|
| Calf Starter[1] | | |
| DM, g/kg | 86.8 | 1.87 |
| Crude protein | 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 |
| Solids non-fat | 10.98 | 1.22 |
| Milk urea nitrogen | 6.00 | 2.86 |

714 [1]Ingredients of calf starter: Rolled corn, canola pellets, rolled barley, molasses, soybean meal,
715 cottonseed hulls.

716

**Table 2.** Comparison of bodyweight, serum total protein, daily starter intake, mortality, and removal rate for treated groups of calves.

| | | Treatment[1] | | | |
|---|---|---|---|---|---|
| Item | n | 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[a] ± 0.38 | 70.7[ab] ± 0.38 | 69.9[b] ± 0.38 | 71.2[ab] ± 0.38 |
| Final BW (56 d), kg | 1,602 | 80.9[ab] ± 0.41 | 81.5[a] ± 0.41 | 80.3[b] ± 0.41 | 81.9[a] ± 0.42 |
| 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% |

n = Number of calves included in model

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

Each value is ± SE.

[1]Treatment: PRO = 14mL Yeast culture + Mannan-oligosaccharide; PRO = 1g *Bacillus subtilis*; SYN = Combination of PRO and PRE. Reported as marginal least squares mean.

725 **Table 3.** Daily bodyweight gain providingleast square means of treatments and model
726 coefficients.

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

727 n = Number of calves included in model.
728 [a–d]Means within a row with different superscripts differ ($P < 0.05$).
729 Each value is ± SE.
730 [1]Treatments: PRE = 14mL Yeast culture + Mannan-oligosaccharide; PRO = 1g *Bacillus subtilis*; SYN
731 =Combination of PRE and PRO. Reported as marginal least squares mean predictions.
732 [2]All listed coefficients at $P<0.05$.
733

734 **Table 4.** Mixed negative binomial regression for length of the first diarrhea episode (d)
735 providing least square means of fecal pathogen shedding for treatment.

| | | Treatment[1] | | | |
|---|---|---|---|---|---|
| Item | n | Control | PRE | PRO | SYN |
| Diarrhea length, d[2] | 1,693 | 4.38±1.03 | 4.41 ±1.03 | 4.27±1.03 | 4.36±1.03 |
| Cryptosporidium, oocysts/g | | | | | |
| 7 d[3] | 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[4] | 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 – 139410 | 44,182 – 1,240,630 |
| 21 d[3] | 160 | 785[ab] | 2201[a] | 395[ab] | 280[b] |
| 95% CI | | 214 – 2,870 | 610 – 7,940 | 108 – 1,446 | 79 – 995 |
| *Escherichia coli*,cfu/g | | | | | |
| 7 d[5] | 265 | $7.7 \times 10^{7ab}$ | $7.6 \times 10^{7\ ab}$ | $1.03 \times 10^{8\ a}$ | $4.4 \times 10^{7\ b}$ |
| 95% CI | | $3.8 \times 10^{7} – 1.17 \times 10^{8}$ | $3.7 \times 10^{7} – 1.15 \times 10^{8}$ | $5.1 \times 10^{7} – 1.15 \times 10^{8}$ | $2.3 \times 10^{7} – 6.6 \times 10^{7}$ |
| 21 d[6] | 243 | $1.7 \times 10^{7}$ | $2.7 \times 10^{7}$ | $1.8 \times 10^{7}$ | $1.8 \times 10^{7}$ |
| 95% CI | | $3.9 \times 10^{6} – 3.1 \times 10^{7}$ | $5.6 \times 10^{6} – 4.8 \times 10^{7}$ | $4.6 \times 10^{6} – 3.2 \times 10^{7}$ | $4.1 \times 10^{6} – 3.1 \times 10^{7}$ |
| 42 d[7] | 267 | $1.6 \times 10^{7a}$ | $7.6 \times 10^{6\ b}$ | $1.3 \times 10^{7ab}$ | $1.4 \times 10^{7ab}$ |
| 95% CI | | $8 \times 10^{6} – 2.4 \times 10^{7}$ | $3.5 \times 10^{6} – 1.1 \times 10^{7}$ | $7 \times 10^{6} – 1.9 \times 10^{7}$ | $7 \times 10^{6} – 2.1 \times 10^{7}$ |
| Pathogenic *E. coli*, cfu/g | | | | | |
| 7 d[8] | 259 | $4.2 \times 10^{7}$ | $4.8 \times 10^{7}$ | $6.3 \times 10^{7}$ | $3.3 \times 10^{7}$ |
| 95% CI | | $1.5 \times 10^{7} – 6.7 \times 10^{7}$ | $1.9 \times 10^{7} – 7.6 \times 10^{7}$ | $2.6 \times 10^{7} – 1 \times 10^{8}$ | $1.3 \times 10^{7} – 5.2 \times 10^{7}$ |
| 21 d[3] | 240 | $2 \times 10^{7}$ | $2.6 \times 10^{7}$ | $2.6 \times 10^{7}$ | $1.7 \times 10^{7}$ |
| 95% CI | | $0 – 4.1 \times 10^{7}$ | $0 – 5.3 \times 10^{7}$ | $0 – 5.3 \times 10^{7}$ | $0 – 3.5 \times 10^{7}$ |
| 42 d[5] | 257 | $1 \times 10^{7\ a}$ | $3.9 \times 10^{6\ b}$ | $6 \times 10^{6\ ab}$ | $9 \times 10^{6\ ab}$ |
| 95% CI | | $4.2 \times 10^{6} – 1.6 \times 10^{7}$ | $1.6 \times 10^{6} – 6.3 \times 10^{6}$ | $2.7 \times 10^{6} – 9.8 \times 10^{6}$ | $3.6 \times 10^{6} – 1.5 \times 10^{7}$ |

| | | | | | |
|---|---|---|---|---|---|
| *Clostridium spp., cfu/g* | | | | | |
| 7 d[9] | 285 | $4.4 \times 10^6$ | $6.8 \times 10^6$ | $5.3 \times 10^6$ | $3.9 \times 10^6$ |
| 95% CI | | $9 \times 10^4 – 8.7 \times 10^6$ | $2.7 \times 10^5 – 1.3 \times 10^7$ | $1 \times 10^5 – 1 \times 10^7$ | $7.3 \times 10^4 – 7.8 \times 10^6$ |
| 21 d[5] | 258 | $5.7 \times 10^4$ | $2.1 \times 10^4$ | $3.9 \times 10^4$ | $5.3 \times 10^4$ |
| 95% CI | | $1 \times 10^4 – 1 \times 10^5$ | $3.9 \times 10^3 – 3.8 \times 10^4$ | $7.2 \times 10^3 – 7 \times 10^4$ | $1 \times 10^4 – 9.6 \times 10^4$ |
| 42 d[5] | 266 | $1.3 \times 10^4$ | $1.4 \times 10^4$ | $1.1 \times 10^4$ | $1.2 \times 10^4$ |
| 95% CI | | $0 – 2.7 \times 10^4$ | $0 – 2.9 \times 10^4$ | $0 – 2.3 \times 10^4$ | $0 – 2.5 \times 10^4$ |
| *C. perfringens,* cfu/g | | | | | |
| 7 d[5] | 284 | $3.9 \times 10^6$ | $1.2 \times 10^7$ | $7 \times 10^6$ | $3.6 \times 10^6$ |
| 95% CI | | $0 – 1.1 \times 10^7$ | $0 – 3.5 \times 10^7$ | $0 – 2 \times 10^7$ | $0 – 1 \times 10^7$ |
| 21 d[10] | 255 | $3.8 \times 10^5$ | $2 \times 10^5$ | $1.9 \times 10^5$ | $4.6 \times 10^5$ |
| 95% CI | | $0 – 9.5 \times 10^5$ | $0 – 5.52 \times 10^5$ | $0 – 4.7 \times 10^5$ | $0 – 1 \times 10^6$ |
| 42 d[3] | 263 | $3.4 \times 10^4$ | $5.3 \times 10^4$ | $3.2 \times 10^4$ | $3.6 \times 10^4$ |
| 95% CI | | $0 – 8.6 \times 10^4$ | $0 – 1.3 \times 10^5$ | $0 – 7.9 \times 10^4$ | $0 – 8.8 \times 10^4$ |

n = Number of calves included in model.

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: PRE = 14mL Yeast culture + Mannan-oligosaccharide; PRO = 1g *Bacillus subtilis*; SYN = Combination of PRE and PRO.

[2]Model included total protein above or below 5.5 g/dL as a fixed effect and calf as a random effect

[3]Model includedcalf as a random effect

[4]Model included block and interaction term between block and treatment as fixed effects and calf as a random effect

[5]Model included month of birth as a fixed effect and calf as a random effect

[6]Model includeddays of age at first diarrhea as a fixed effect and calf as a random effect

[7]Model included month of birth, total protein above or below 5.5 g/dL and birth weight as fixed effects

[8]Model 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 and calf as a random effect

[9]Model included month of birth and total protein above or below 5.2 g/dL as fixed effects and calf as a random effect

[10]Model included month of birth, body weight and total protein above or below 5.2 g/dL as fixed effects and calf as a random effect

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

| Item | n | Treatment[1] | | | |
|---|---|---|---|---|---|
| | | Control | PRE | PRO | SYN |
| Diarrhea, Hazard ratio | 1,797 | Referent | 1.01 ± 0.07 | 0.96 ± 0.07 | 1.02 ± 0.07 |

n = Number of calves included in model.

[a–d] Means within a row with different superscripts differ ($P$ <0.05).

[1]Treatments: PRE = 14mL Yeast culture + Mannan-oligosaccharide; PRO = 1g *Bacillus subtilis*; SYN = Combination of PRE and PRO.

764 **Table 6.** Odds ratio of at least one pneumonia event

| Item | n | Treatment[1] | | | | Model coefficients[2] |
|---|---|---|---|---|---|---|
| | | Control | PRE | PRO | SYN | Birth BW, kg |
| Pneumonia, Odds Ratio | 1,758 | Referent | 0.82 ± 1.03 | 0.95 ± 0.17 | 0.82 ± 0.15 | 1.03 ± 0.01 |

765 n = Number of calves included in model
766 [1]Treatments: PRE = 14mL Yeast culture + Mannan-oligosaccharide; PRO = 1g *Bacillus subtilis*;
767 SYN = Combination of PRE and PRO.
768 [2]All listed coefficients at $P<0.05$.
769

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