Addition of a *Bacillus* based probiotic to the diet of preruminant calves: Influence on growth, health, and blood parameters[1,2,3]

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ABSTRACT

This study evaluated the addition of a Bacillus-based probiotic to milk replacer and starter for preruminant calves. Forty Holstein calves (1 to 4 d old) were housed individually and blocked by sex and date of birth with treatments assigned randomly within blocks. The treatments were probiotic (PRO; 10^9 cfu/d) added to the milk replacer or control (CON) in which there was no additive. All calves received a milk-based milk replacer containing their respective treatment during the initial 14 d on the experiment. On d 15, treatment was maintained but milk replacer was abruptly switched from milk to soy based. All calves remained on the soy-based milk replacer until weaning. Weaning occurred when starter consumption exceeded 1% of body weight for three consecutive d. After 42 d on the study, unweaned calves were reduced to one feeding of milk replacer daily to promote increased starter intake. Weaned calves were maintained on a commercial starter containing probiotic at 10^6 cfu/g starter (PRO) or starter with no microbial additives (CON) ad libitum through the 56-d experiment. Dry matter intake (sum of the two food sources) was recorded daily and fecal output was scored (fecal scoring: fluidity, 1=normal, 2=soft, 3=runny, 4=watery; Consistency, 1 = normal, 2 = foamy, 3 = mucus, 4 = sticky, 5 = constipated; Odor, 1 = normal, 2 = slightly offensive, 3 = highly offensive). A scour day was recorded if fluidity = 3 or 4, consistency = 3, and odor = 2 or 3. Calves were weighed weekly and measured for hip height, wither height, hip width and heart girth. Blood samples were collected weekly by jugular venapuncture and analyzed for hematocrit, plasma Beta-hydroxybutyrate (BHB), total protein (TPROT), and Immunoglobulin G1 (IgG1) concentrations. Treatment did not affect d to weaning, incidence or duration of scouring, or growth performance ($P \geq 0.27$). Hematocrit and plasma TPROT and IgG1 concentrations changed ($P \leq 0.002$) over time

Table 1: *Composition of milk replacer (milk & soy based) and starter.*

Nutrient	min/max	Milk Replacer[ab]	Starter[cd]
Crude Protein, %	min	22	18
Crude Fat, %	min	20	2
Crude Fiber, %	max	0.15	8
Ca, %	min	0.75	-
Ca, %	max	1.25	1.45
P, %	min	0.70	0.62
Se, ppm	-	-	0.40
Vit A, IU/kg	min	9,080	5,900
Vit D3, IU/kg	min	2,270	-
Vit E, IU/kg	min	45.40	-
Decoquinate, %	-	-	0.00333

[a] *Instant Amplifier Max Dairy herd & beef calf milk replacer (milk based); Instant Maxi Care Dairy herd & beef calf milk replacer (soy based; Land O Lakes Animal Milk Products, Co., Shoreview, MN)*
[b] *Milk replacer + Bioplus 2B (PRO; 10^9 cfu/d; Chr. Hansen, Inc. Milwaukee, WI)*
[c] *Calf Developer Plus Medicated (Southern States Cooperative, Inc., Richmond, VA)*
[d] *Starter + Bioplus 2B (PRO; 10^6 cfu/g starter)*

but were unaffected by treatment ($P \geq 0.31$). Plasma BHB concentrations increased ($P < 0.0001$) over time and tended ($P = 0.12$) to be greater for PRO calves. However, lack of difference in growth performance and health characteristics may indicate that calves housed indoors in a temperature controlled environment with little added stressor may not benefit from probiotic feeding.

INTRODUCTION

Interest in the effects of feeding probiotics on animal health and performance has increased due to concern regarding the use of antibiotics and other growth stimulants in the animal feed industry.[1] This concern has increased the emphasis placed on disease prevention as a means of reducing the use of antibiotics and lessening public fears about antibiotic residues in meat and meat products.

Generally, the importance of feeding probiotics to neonatal livestock has been to establish and maintain normal intestinal microorganisms rather than as a production stimulant.[2] In the neonate, the microbial population of the gastrointestinal tract (GIT) is in transition and extremely sensitive.[1] Abrupt environmental or dietary changes may cause shifts in the microbial population of the GIT which often leads to an increased incidence of diarrhea in calves.[3] Gastrointestinal disorders, including diarrhea, are one of the leading causes of mortality and morbidity in neonatal calves, and a reduction in the incidence and duration of diarrhea has been reported in calves consuming probiotics.[4,5] In addition to decreasing the occurrence of diarrhea, some studies have indicated that inclusion of probiotics in the diet improves weight gain, feed efficiency, and feed intake.[6-8] However, these results are in contrast with those who have not observed benefits from feeding probiotics.[9,10]

Lactic acid bacteria (LAB), including members of the genera Lactobacillus and Enterococcus (formerly Streptococcus), are the most extensively studied probiotics. This is largely due to the natural occurrence of these bacteria in the healthy GIT.[11] Although the mechanisms through which probiotics assert their benefits have not been identified, lactic acid bacteria are thought to inhibit the growth of pathenogenic bacteria by lowering pH in the GIT though production of lactic acid and through competitive attachment.[12,2] Non-pathenogenic Bacillus spore forming

species including B. subtilis and B. lichenformis may be included as one or more of the components in some products.[13] Bacillus spores such as B. subtilis and B. lichenformis are not normal inhabitants of the GIT and may stimulate the immune system due to the presence of these unharmful spores.[14] Bacillus strains like those in the commercial product Bioplus 2B have also been shown to produce antimicrobials.[15]

This study evaluated the addition of a Bacillus based probiotic to milk replacer and starter for calves and its effects on growth, health, and blood parameters.

MATERIALS AND METHODS

All experimental procedures used were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals and Treatments

Forty Holstein bull (n=20) and heifer (n=20) calves (2 to 4 d old) were received from the University of Kentucky dairy herd over a six-month period. Immediately following birth, calves received colostrum, fed at 10% of body weight divided in two equal meals during the first 12 h after birth, and navels were dipped in 7% iodine. Calves were transported to the Animal Lab in the basement of the W.P. Garrigus building on the campus of the University of Kentucky. Calves were housed individually in solid-sided pens (2.6 x 2.4 m) in a climate controlled room (21 - 27°C) with a 12 h light cycle and continuous access to water. Calves were fed a common diet of milk-based milk replacer (Table 1; 340 g milk replacer powder mixed with 2 L water fed every 12 h). A commercially available starter (Table 1) and water were available ad libitum.

Calves were assigned by sex and birth date to 20 blocks containing 2 calves each in a randomized complete block design. Calves were assigned randomly to treatment within each block. Calves were allotted to treatments twice weekly to enable a common starting date and to ensure that calves were started on treatment by 4 d of age. The treatments included probiotic, BioPlus 2B (PRO; B. subtilis + B. lichenformis; Chr. Hansen, Inc, Milwaukee, WI), added to the milk replacer at a level of 10^9 cfu/d or control (CON) in which there was no additive to the milk replacer. Each animal had individual bottles to avoid cross contamination. In addition to milk replacer, PRO treated calves were supplied a commercial starter (Table 1) ad libitum containing Bioplus 2B (10^6 cfu/g starter) while CON calves received the same starter with no additives.

All calves received a milk-based milk replacer containing the respective treatments during the initial 14 d on the experiment. On d 15 calves were abruptly switched to a soy-based milk replacer containing their respective treatment. The abrupt change in milk replacer was intended to induce stress to the GIT. All calves remained on the soy-based milk replacer until weaning. Starter consumption was monitored daily, and weaning occurred when starter consumption exceeded 1% of body weight for 3 consecutive d. After d 42 d, unweaned calves were reduced to one feeding of milk replacer per d to promote starter intake. Following weaning, calves were offered starter containing their respective treatments at ad libitum intake for the remainder of the 56-d experiment.

The amount of feed offered and orts were recorded daily throughout the experiment. The diet (both milk replacer and starter) was sampled daily, composited weekly, and analyzed for dry matter. Dry matter was determined by placing samples in a 100°C convection oven for 24 h (Thelco Lab Oven, Precision Scientific, Winchester, VA).

Fecal output was observed and scored daily (fecal scoring: fluidity, 1 = normal, 2 = soft, 3 = runny, 4 = watery; Consistency, 1 = normal, 2 = foamy, 3 = mucus, 4 = sticky, 5 = constipated; Odor, 1 = normal, 2 = slightly offensive, 3 = highly offensive). A scour d was recorded if the fluidity score was 3 or 4, consistency was 2 or 3, and odor scored a 3. Calves were weighed weekly and measured for hip height, wither height, hip width and heart girth.

Blood collection and plasma analysis

Blood samples were collected weekly by

jugular venapuncture 3 h post feeding for analysis of hematocrit, total protein (TPROT), Immunoglobulin G1 (IgG1) and Beta-hydroxybutyrate (BHB) concentrations. The blood was collected into a BD Vacutainer (Benton Dickinson, Franklin Lakes, NJ) containing sodium heparin, inverted, and placed on ice. The whole blood was analyzed in duplicate for hematocrit by centrifugation (3 minutes) of capillary tubes (Autocrit Utra3, Clay Adams, Parsippany, NJ), and the packed cell count was then read using an International Micro-Capillary Reader (International Equipment Co., Needham Heights, MA). The remaining whole blood was centrifuged (Sorvall Instruments RT6000B, DuPont, Newtown, CT) at 1700 x g for 15 minutes at 10°C. The resulting plasma was transferred into a scintillation vial and stored frozen (-20° C) until further analysis. After plasma samples were collected for all calves and all time points, the frozen plasma samples were thawed and analyzed for TPROT and BHB using a Konelab 20 XTi (Thermo Electron Corp., Vantaa, Finland). Total protein was determined using quantitative calorimetric determination (Stanbio Total Protein Liquicolor Procedure Number 0250; Stanbio Laboratory, Boeme, TX). Beta-hydroxybutyrate concentration was determined using Beta-Hydroxybutyrate dehydrogenase (Stanbio LiquiColor Procedure Number 2440). Plasma IgG1 concentration was analyzed using an ELISA quantification kit (Bethyl Laboratories, Inc., Montgomery, TX).

Table 2: *Effect of probiotic on performance variables in neonatal calves.*

	Treatment			
Item	Probiotic	Control	SEM[a]	P value[b]
Days to wean	39.15	39.05	1.65	0.97
Dry Matter Intake, kg				
Pre-Wean	30.77	30.44	1.36	0.87
Post-Wean	32.21	30.39	4.09	0.76
Overall	62.98	60.83	2.97	0.62
Total Weight Gain, kg	23.14	22.28	1.81	0.74
Average Daily Gain, kg				
Pre-weaning	0.28	0.26	0.02	0.50
Post-weaning	0.62	0.60	0.07	0.89
Overall	0.41	0.40	0.03	0.74
Feed:Gain				
Pre-weaning	3.65	3.55	0.25	0.78
Post-weaning	2.89	3.59	1.17	0.68
Overall	3.09	2.83	0.20	0.36
Change in Heart Girth, cm	12.80	13.53	0.78	0.52
Change in Wither Height, cm	2.90	2.71	0.57	0.27
Change in Hip Height, cm	8.00	7.94	0.86	0.96
Change in Hip Width, cm	2.90	2.71	0.20	0.49
Scour Days/calf	5.45	4.65	0.85	0.51

[a]*Pooled standard error of the mean, n=20 calves per treatment.*
[b]*Probability of a larger F statistic.*

Statistical Analysis

All statistical analyses were conducted using the mixed model procedure of SAS (SAS Inst. Inc., Cary, NC). Data were analyzed as a randomized complete block design with calf as the experimental unit. Block was each set of two calves accumulated (same sex). The model included treatment and time with calf and block being random effects. Effect of time and the interaction between treatment and time were included in the model as fixed effects when blood metabolites and hematorcrit were analyzed. All data are presented as means. No interactions between treatment and time were detected ($P>0.10$). Treatment effects were considered significant at $P \leq 0.05$ and as a tendency at $P \leq 0.15$.

RESULTS

Growth performance and feed intake variables are presented in Table 2. Inclusion of probiotic to the diet of calves did not affect d to weaning or scour d per calf ($P\geq0.51$). There was also no treatment effects on dry matter intake, pre-weaning, post-weaning, overall average daily gain, or changes in heart girth, hip width, wither or hip height ($P\geq0.27$). There were no treatment differences in preweaning or overall feed:gain ($P\geq0.36$).

No treatment x time interactions were noted for hematocrit, plasma BHB or TPROT ($P>0.31$). Blood hematocrit ($P=0.03$; Figure 1) concentration was influenced by d, however, no discernable pattern was observed over the experimental period. Plasma total protein (Figure 2) declined from d 0 to d 14 and remained at nadir levels through d 28; after which it returned to near d 0 levels by d 49 ($P<0.0001$). Plasma BHB concentration (Figure 3) was similar during the initial 14 d of the experiment, but increased in a linear fashion from d 21 to d 56 ($P<0.0001$). Although plasma IgG1 concentration changed over time ($P<0.0004$), there was a tendency ($P=0.15$) for a time x treatment interaction (Figure 4). Plasma IgG1 concentrations tended to be greater for probiotic treated calves on d 0 ($P=0.10$) and d 42 ($P=0.13$), but was unresponsive ($P\geq0.20$) to treatment at other time points. Blood hematocrit and plasma TPROT concentrations were unresponsive ($P\geq0.52$) to addition of probiotic to the diet. However, plasma BHB concentrations tended to be higher ($P=0.12$) for PRO treated calves (Figure 1).

DISCUSSION

Previous research in the use of probiotics in pre-ruminant calves has lacked consistent results. Inclusion of probiotics in the diet of young calves has been shown to improve performance characteristics including body weight gain and feed conversion as well as average daily gain in the first two weeks of life.[7,16,10] Moreover, probiotic treated animals have exhibited a reduced incidence and duration of diarrhea.[7,17,16] Contrariwise there is much research which shows no benefit.[9,18,8,19]

In the current study, no treatment differences were noted in d to weaning. Both treatment groups had an average weaning of about 39 d. Dry matter intake, body weight gain, average daily gain, and feed conversion were also unaffected by inclusion of probiotics to the diet. Jenny et al. observed similar results while administering a B. subtilis probiotic at a similar level to calves of comparable age to the current study.[9] This is in disagreement with Abe et al. who saw increased body weight gain and more efficient feed conversion with probiotic treated calves (7 d old; 56 d study).[7] Using calves similar in age to the ones utilized in the current study, Timmerman et al. saw increases in body weight gain and gain:feed as well as increased average daily gain with inclusion of a probiotic to the diet[16]. Both Abe et al. and Timmerman et al. used comparable levels of probiotic bacteria to the current study, but both studies used lactic acid bacteria based probiotics.[7,16]

No differences were noted in the current study in heart girth, wither height, hip height or width between treatments. These indices, along with BW gain, indicate that overall growth was unaffected by treatment. Similarly, Jenny et al. observed no differences in the same indices with probiotic inclu-

***Figure 1**: Hematocrit values of calves fed diets containing a Bacillus based probiotic (PRO) or no additive (CON). Time (P=0.002) and treatment (P=0.52) effects.*

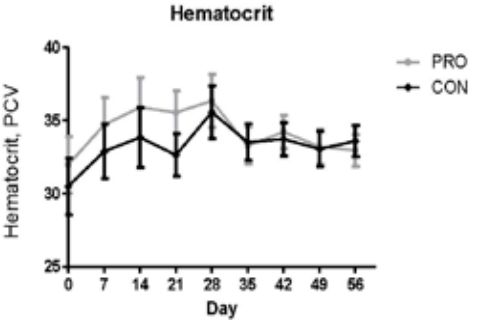


***Figure 3**: Concentration of Beta-hydroxybutyrate in plasma of calves fed diets containing a*
Bacillus based probiotic (PRO) or no additive (CON). Time (P<0.0001) and treatment (P=0.12) effects.

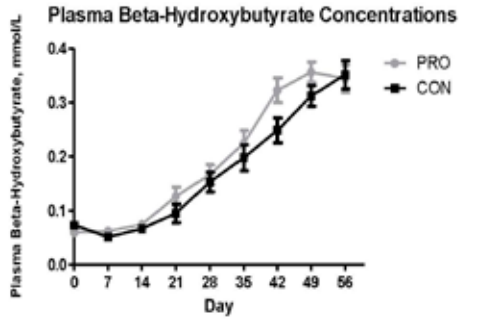


***Figure 2:** Total protein concentration in plasma of calves fed diets containing a Bacillus based probiotic (PRO) or no additive (CON). Time (P<0.0001) and treatment (P=0.58) effects.*

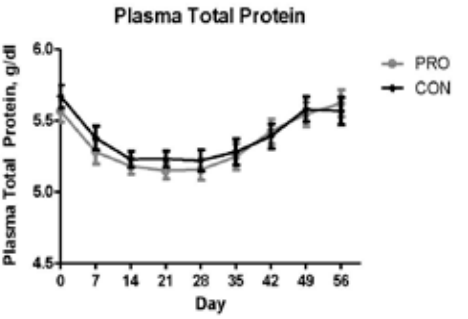
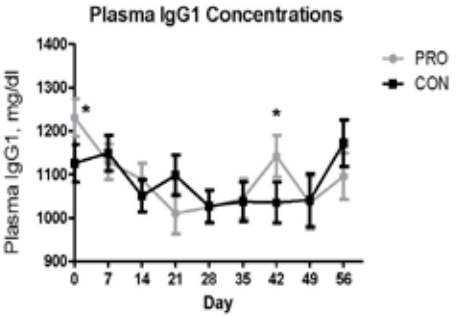


***Figure 4:** Plasma Immunoglobulin G[1] concentrations in calves fed diets containing a Bacillus based probiotic (PRO) or no additive (CON). Time (P=0.0004), treatment (P=0.87), and time x treatment (P=0.15) effects.*
**Tends to differ from CON at d 0 (P=0.10) and d 42 (P=0.13).*



sion.[9] Windschitl et al. saw an increase in heart girth with probiotic treated calves, but remaining measurements were unresponsive to treatment.[19]

No effects on frequency or length of diarrhea or fecal score were observed in the current study. This is in agreement with previously published research.[9,16,8,10] Abe et al. and Abu-Tarboush et al. both saw decreased incidence of diarrhea with inclusion of probiotics to the diet.[7,17] The differences in response may be explained by the type of bacteria used. In the studies that saw favorable results combinations of LAB and Bifidobacterium or Bifidobacterium alone were used whereas in the current study combination of two types of Bacillus spores were utilized.

Since studies using probiotics have been inconsistent in their impacts on growth and health variables, blood metabolites were measured in the current study to determine whether physiological responses to the addition of probiotic to the diet could be detected even if no significant growth or fecal response was observed. No differences were observed between treatments in packed cell volume throughout the study (Figure 1). Adams et al. also found no variation between probiotic treated calves and their

control counterparts in overall hematocrit.[20] Although packed cell volume was influenced by d in the current experiment, hematocrits were within the normal range for both treatments throughout the experiment.[21]

A significant week effect was observed in plasma BHB concentrations as BHB values steadily increased over the course of the study. This increase was expected with increase in intake of fermentable substrate. Metabolically, the neonatal rumen is essentially nonfunctional with respect to ketogenic capacity as it produces negligible amounts of ketones due to the absence of microbial fermentation, and the liver is the primary site of ketogenesis in the preruminant calf.[22,23] Following the initiation of solid feed intake by the neonate and the subsequent establishment of ruminal fermentation, physical and metabolic development of the rumen occurs and the ruminal epithelium becomes the primary source of ketone bodies in fed ruminants.[22,24] About 80% of absorbed butyrate is converted to BHB and some acetoacetate prior to release into portal circulation.[25] Acetoacetate is then removed from circulation and converted to BHB by the liver.[24] Beta-hydroxybutyrate values tended to be higher for probiotic treated calves versus control calves over time. The switch from liver ketogenesis to ruminal ketogenesis may explain this increase in BHB over the course of the study and may indicate that probiotic treated calves had greater ruminal development and were more able to produce ketones more quickly than control calves. This increase in BHB may indicate a greater level of rumen fermentation for probiotic treated calves versus control calves.

Plasma TPROT means did not differ between treatments over the course of the study. This is consistent with the findings of Adams et al. who also found no differences between calves receiving probiotics and control animals.[20] In the current study, however, a time effect was noted indicating a decrease in TPROT through d 28 and then a steady increase through d 56. This shift most likely occurs from the reduction of maternally acquired antibodies over the first weeks of life and the consequential production of antibodies by the calf as the passively acquired maternal antibodies wane.[25] Although initial and d 42 IgG1 concentrations tended higher in PRO treated calves, this did not translate into differences in TPROT. Plasma IgG is highest after birth from passive transfer of colostrum antibodies. The concentration of IgG in the plasma then lowers until an animal is able to produce its own antibodies.[25] Thus the slight elevation in plasma IgG1 concentration for PRO calves on d 0 most likely represents differences in colostrum IgG1 concentration or intake and were independent of treatment. Conversely, the tendency for increased IgG1 concentration for PRO treatment on d 42 was preceded by PRO inclusion. However, the lack of elevated values at other time points makes the interpretation of this single time point increase difficult. It was hypothesized that addition of a Bacillus based probiotic to the diet would stimulate an increase in IgG1 levels as an anti-spore immune response.[13] Using comparable levels of inclusion, Spiehs et al. observed a similar nonresponse in serum IgG1 levels using a B. subtilis probiotic in finishing pigs.[26] Duc et al. indicated an increase in IgG1 levels in mice dosed with B. subtilis.[14] However, it should be noted that the level of B. subtilis administered was almost ten times the level that calves in the current study were dosed. Perhaps the dose of the Bacillus based probiotic used in this study was too low to observe a measurable response in IgG1.

CONCLUSIONS

Probiotics are most effective in times of stress. Thus, it is plausible that the lack of differences between treatments in performance, growth, and health variables may be due to the lack of stress imposed on the calves in the current study. The calves were housed indoors in a temperature controlled environment with adequate ventilation. The study contained a considerable diet change. However, the switch from a milk based to a soy based milk replacer may not have been

substantial enough to stress the calves to the point of dysbiosis. A higher level of induced stress may be necessary to observe greater benefits of adding a Bacillus based probiotic to the diet of calves.

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