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CASE STUDY: Effects of a blend of prebiotics, probiotics, and hyperimmune dried egg protein on the performance, health, and innate immune responses of Holstein calves

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ABSTRACT

The objectives of this study were to evaluate the effects of supplementing a blend of prebiotics, probiotics, and hyperimmune dried egg protein on the performance, health, and innate immune responses of Holstein calves during the first 21 d of age. Ninety, day-old Holstein bull calves were completely randomized to 1 of 2 treatment groups: prophylactic or control. Prophylactic calves had a proprietary blend of prebiotics, probiotics, and hyperimmune egg protein (Calf-Pro Plus and Calf Life, C and E Agri Products, Baldwin, ND) added directly to the milk replacer at each feeding. Control calves were fed the milk replacer only. Treatment did not influence the performance of Holstein calves. Among calves that voluntarily refused milk replacer, prophylactic calves refused less ($P = 0.005$) than control calves over the first 4 d of life. Prophylactic treatment reduced the incidence of enteric morbidity (25.0

*vs. 51.1% for prophylactic and control calves, respectively; $P = 0.011$). However, treatment did not influence either total peripheral leukocyte or differential counts. Furthermore, hematocrit and plasma concentrations of haptoglobin, cortisol, glucose, and urea nitrogen were not different between treatments. Last, *ex vivo* innate immune responses evaluated at 21 d of age were not different between treatments. Calves fed the combination of prebiotics, probiotics, and hyperimmune egg protein rejected less milk replacer during the first 4 d of the experiment and had fewer days with enteric morbidity than did the control calves.*

Key words: calf, egg protein, health, prebiotic, probiotic

INTRODUCTION

The risks of morbidity and mortality among dairy calves due to enteric infections are high. Dairy calf morbidity and mortality were reduced and performance enhanced when the antibiotics oxytetracycline and neo-

mycin were included in milk replacer (Quigley et al., 1997). However, there are concerns that the use of antimicrobials in livestock industries causes an increase in antibiotic-resistant bacteria that cause disease in humans. Therefore, alternatives to the use of such metaphalactic antimicrobials are needed. There is interest in the use of prebiotics, probiotics, and dried egg protein from hyperimmunized laying hens to improve enteric health in calves (Abe et al., 1995; Ikemori et al., 1997; Heinrichs et al., 2003). Probiotics are live, nonpathogenic microorganisms that colonize the gastrointestinal tract, whereas prebiotics are nondigestible dietary components that stimulate microorganism growth in the gastrointestinal tract. Immunizing laying hens against various epitopes on pathogenically relevant microorganisms increases the concentration of IgY-specific antibodies in whole dried egg (Hennig-Pauka et al., 2003).

Influences of prebiotics and probiotics on the health of dairy calves are equivocal. Some research showed re-

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duced scouring and improved performance (Abe et al., 1995; Heinrichs et al., 2003), whereas other data showed no benefits from including either prebiotics or probiotics in milk replacer (Morrill et al., 1995; Hill et al., 2008). Inclusion of whole dried egg from hyperimmunized hens decreased morbidity due to various bacteria (Henning-Pauka et al., 2003) and viruses (Kuroki et al., 1993; Ikemori et al., 1997). The objectives of the current study were to evaluate the effects of supplementing a blend of prebiotics, probiotics, and hyperimmune dried egg protein on the performance, health, and innate immune responses of Holstein calves during the first 3 wk of life.

MATERIALS AND METHODS

Animals, Housing, and Treatments

All animal care and use was approved by the Animal Care and Use Committee at Texas Tech University. The experiment was conducted in February 2010. Ninety Holstein bull calves (12 to 36 h after birth) were acquired daily from 2 local commercial dairy herds over a 7-d period. All calves were fed 3.8 L of pooled colostrum from each dairy within 12 h of birth. All calves were transported approximately 60 km to the Hilmar Cheese Calf Research Facility at Texas Tech University in New Deal, Texas. Calves were housed individually in commercial polyethylene calf hutches (Agri-Plastics, Tonawanda, NY) that were bedded with straw.

Upon arrival at the research facility, all calves were weighed and 10 mL of peripheral blood was collected by jugular venipuncture for quantification of total serum protein using a temperature-controlled refractometer. Within dairy source, calves were completely randomized to 1 of 2 treatment groups, which included a negative control (control) and a prophylactic treatment (prophylactic) (n = 45 calves/treatment group). After assignment of treatments, the prophylactic calves were administered a one-time

oral bolus (100 mL) of 7.5×10^8 total cfu from a combination of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium thermophilum*, *Enterococcus faecium*, and *Bifidobacterium longum*. Control calves were not given an oral bolus of probiotics. All calves were offered 227 g of powder (as-fed) of a nonmedicated 20% CP and 20% fat milk replacer (HerdMaker, Land O'Lakes Animal Milk Products Co., Shoreview, MN) twice daily at 0800 and 1600 h. Prophylactic calves were further administered 5×10^9 total cfu/d from a combination of *L. acidophilus*, *B. subtilis*, *B. thermophilum*, *E. faecium*, and *B. longum*; 2 g/d of a proprietary blend of mannan-oligosaccharides, fructo-oligosaccharides, and activated charcoal; and 3.2 g/d of dried egg protein from laying hens vaccinated against K99+ *Escherichia coli* antigen, *Salmonella typhimurium*, *Salmonella dublin*, coronavirus, and rotavirus added directly to the milk replacer (CalfPro Plus and Calf Life, C and E Agri Products, Baldwin, ND). The daily prophylactic treatment was divided in half and administered at each feeding. Control calves were not administered any prebiotics or probiotics or dried egg protein. Prophylactic calves with consecutive days with fecal scores ≥ 3 were given acidified oral electrolytes added directly to the milk replacer and were given an additional dose of 7.5×10^8 total cfu/d from a combination of *L. acidophilus*, *B. subtilis*, *B. thermophilum*, *E. faecium*, and *B. longum*. Control calves with consecutive days with fecal scores ≥ 3 were treated with acidified oral electrolytes and 16 mg of Trimethoprim (Pfizer Animal Health, New York, NY) and 3.2 mg of Sulfa (Pfizer Animal Health) per kilogram of BW added directly to the milk replacer. The administration of this antibiotic combination to morbid calves is the standard operating procedure for dairy calves during the first 3 wk of life at the Texas Tech University calf facility. A calf with consecutive days with fecal scores ≥ 3 was classified as a morbid calf. All other feeding and management strategies were similar between the 2 treat-

ment groups. After the first week all calves were offered ad libitum access to a calf starter (Table 1) and water. Daily intakes of calf starter were calculated from the quantity of starter offered minus daily refusal. Samples of calf starter were collected every Monday, Wednesday, and Friday and composited by week for analyses of DM. Estimates of the DM of refusals were also collected from a composite of the calves on Monday, Wednesday, and Friday. The DM measurements of both the calf starter and refusals were used to calculate the DMI of calf starter.

Sampling

Milk replacer refusals were recorded from each calf 30 min after each feeding. Research personnel considered refusals when the calf would not suckle anymore. Calf starter intake was measured daily. Fecal scores were classified by 2 independent trained observers multiple times daily according to the guidelines outlined by Larson et al. (1977). Briefly, 1 = firm, well-formed (not hard); 2 = soft, pudding-like; 3 = runny, pan-

Table 1. Calf starter ingredients and chemical composition

Item	Amount
Ingredient, % DM	
Steam-flaked corn	67.3
Soybean meal, 48%CP	13.3
Cottonseed meal	11.3
Molasses	5.3
Calf mineral-vitamin premix ¹	2.8
Chemical composition	
DM, %	88.2
CP, %	18.9
ME, Mcal/kg	3.06

¹Supplement contained (DM basis) 52.6% limestone; 39.9% soybean meal; 6% salt; 0.451% zinc sulfate; 0.4% selenium selenite 0.2%; 0.267% manganese oxide; 0.18% vitamin E, 500 IU/g; 0.157% copper sulfate; 142 mg/kg vitamin A, 1,000 KIU/g; 12.5 mg/kg ethylenediamine dihydroiodine; 8.7 mg/kg cobalt carbonate.

cake batter; and 4 = liquid, splatters, pulpy orange juice. On 7 and 21 d of age, each calf was weighed before the AM feeding, and on 7, 14, and 21 d of age, each calf had 9 mL of peripheral blood collected via jugular venipuncture into 2 heparinized vacutainers (3 and 6 mL) at 0700 h. On 21 d of age, a completely random subset of 35 calves from each treatment group had an additional 9 mL of peripheral blood collected for ex vivo analyses of innate immune responses. A subset of 35 calves per treatment allowed all samples to be processed within one day and have an 80% protection against a Type II error.

Blood and Plasma Analyses

Within 1 h of collection, whole blood was analyzed for hematocrit, total leukocyte counts, and differentiation for neutrophils and mononuclear cells using a Cell Dyn 3700 with an automated 50-sample loader and veterinary software package (Abbot Laboratories, Abbot, IL). In addition, the neutrophil:mononuclear cell ratio was calculated. Plasma was collected after centrifugation at $1,250 \times g$ and stored at -80°C until analyses for cortisol, glucose, urea nitrogen, and haptoglobin concentrations. All plasma samples were analyzed in duplicate. Plasma concentrations of cortisol were determined using a commercially available ELISA (Arbor Assays, Ann Arbor, MI); intraassay and interassay CV were 4.5 and 5.2%, respectively. Plasma concentrations of glucose and urea nitrogen concentrations were analyzed using commercially available enzymatic kits (Stanbio Laboratory, Boerne, TX), and intra- and interassay CV for these assays were 3.9 and 4.5%, respectively. Plasma haptoglobin concentrations were determined by measuring haptoglobin/hemoglobin complex by the estimation of the difference in peroxidase activity (Makimura and Suzuki, 1982; Arthington et al., 2003). Results are expressed in arbitrary units resulting from the absorbance at $450 \text{ nm} \times 100$. The intra- and interassay CV were 1.7 and 2.0%, respectively.

Ex Vivo Immunological Assays

All ex vivo immunological methods were described by Hulbert et al. (2011). Briefly, the simultaneous phagocytic and oxidative burst capacities of circulating neutrophils were evaluated in response to an enteropathogenic *E. coli* isolated from the spleen of a septicemic calf. Data are reported as the percentage of neutrophils phagocytizing and producing an oxidative burst as well as their geometric fluorescence intensities. In addition, the expression of the adhesion molecules, L-selectin and β_2 -integrin, on circulating neutrophils was determined. Data are reported as their geometric mean fluorescence intensities. Finally, whole blood was cocultured at a final concentration of $1 \mu\text{g/mL}$ of lipopolysaccharide (*E. coli* 0111:B4; Sigma-Aldrich, St. Louis, MO) for 24 h. Following the incubation, cell cultures were centrifuged at $1,200 \times g$ at 4°C and supernatants were collected and stored at -80°C until analysis for concentrations of tumor necrosis factor- α using a commercially available ELISA (R & D Biosystems, Minneapolis, MN). The intraplate CV was 3.8%.

The innate immune responses evaluated in the current study were chosen because they reflect key responses in the recognition (whole blood tumor necrosis factor- α response), recruitment (expression of adhesion molecules L-selectin and β_2 -integrin), and elimination of bacteria (phagocytic and oxidative burst capacities to an enteropathogenic *E. coli*).

Statistical Analyses

Repeated, continuous data were analyzed by restricted maximum likelihood ANOVA using the Mixed procedure of SAS (SAS version 9.2, SAS Inst. Inc., Cary, NC). The model included treatment, day, and treatment \times day as the fixed effects. Calf nested within treatment was the random effect. The appropriate covariance structure for the within-calf measurements was chosen based on the Schwartz Bayesian criterion.

Nonrepeated, continuous data were analyzed by ANOVA using the general linear procedure of SAS with treatment as the fixed effect. Furthermore, only calves that were classified as scouring (consecutive fecal scores ≥ 3) were analyzed to determine the initiation and duration of scours. All data analyzed by ANOVA were tested for homogeneity of variance and normality using the Univariate procedure of SAS.

Enteric morbidity and milk replacer refusal were analyzed using the chi-squared probability test using the Frequency procedure of SAS. In addition, because there was no difference between treatments in the frequency of calves refusing milk replacer from d 1 to 4 after enrollment, the sum of milk replacer refusal from only calves that had a milk replacer refusal from d 1 to 4 was analyzed by the Wilcoxon rank test using the NPAR1WAY procedure of SAS. Throughout the manuscript, treatment differences of $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered tendencies. Least squares means (\pm SEM) are reported throughout unless otherwise noted.

RESULTS AND DISCUSSION

The influences of supplementing a blend of prebiotics, probiotics, and hyperimmune dried egg protein on the performance, health, and innate immune responses of Holstein calves during the first 21 d of age were investigated. The study was conducted in February 2010. The average low and high temperatures during the study period were -1.8 and 9.8°C , respectively. At enrollment there were no differences in initial BW or total serum protein between treatments (Table 2). There were no differences observed in the performance of calves between treatments (Table 2). Among all calves, there was a negative ADG during the first week, -0.261 kg/d , which was then positive from d 7 to 21, 0.426 kg/d . These results are similar to those of Hill et al. (2008), wherein they observed no difference in ADG from birth through wean-

Table 2. Effect of prophylactic blend of prebiotics, probiotics, and hyperimmunized egg protein on the performance of neonatal Holstein calves

Item	Treatment (Trt)			Main effect, <i>P</i> <		
	Control	Prophylactic	Largest SEM	Trt	Time	Trt × time
n	44	45				
Initial serum protein, g/dL	4.72	4.97	0.147	0.201	—	—
Initial BW, kg	42.65	42.71	0.741	0.954	—	—
ADG, kg/d	0.221	0.174	0.0374	0.504	0.001	0.408
Voluntary milk refusal from d 1 to 4, ^{1,2} kg of DM	0.149	0.057	—	0.005	—	—
Starter intake, kg of DM	2.59	2.43	0.209	0.579	—	—
G:F, kg of BW/kg of DM	0.106	0.123	0.0348	0.737	—	—

¹There was no difference in the frequency of calves refusing milk between treatments (57 vs. 51% for control and prophylactic, respectively; *P* = 0.589).

²Reported as medians and significance from Kruskal–Wallis test.

ing when calves were supplemented with either 4 or 8 g/d of a prebiotic fructo-oligosaccharide (Ultra-FOS, Encore Technologies LLC, Minneapolis, MN). In addition, Heinrichs et al. (2003) did not observe any differences in ADG when Holstein calves were supplemented with 4 g/d of a prebiotic (Bio-Mos, Alltech, Nicholasville, KY); however, they did observe a postweaning increase in calf starter intake among calves fed the prebiotics. In contrast to the present data, Abe et al. (1995) observed increases in ADG and efficiency of BW gains when Holstein calves were either administered 3×10^9 cfu/d of the probiotic *Bifidobacterium pseudolongum* M-602 or *L. acidophilus* LAC-300 in the milk replacer. In addition, Cruywagen et al. (1996) reported that supplementing milk replacer with 5×10^7 cfu/d of *L. acidophilus* prevented the loss in BW that is often observed in dairy calves during the first 1 to 2 wk of life. In contrast, Morrill et al. (1995) did not observe any increase in performance when Holstein calves were supplemented with a probiotic (Biomate, Chr. Hansen, Milwaukee, WI); however, they did observe an increase in performance when a portion of the protein in the milk replacer was replaced with spray-dried plasma from either porcine or bovine sources. In contrast, when Quigley et al. (2002) replaced either 16 or 20% of the CP

in the milk replacer with spray-dried bovine plasma, they did not see any improvements in ADG from 0 to 28 d of age. The present data, when taken together with past literature, indicate that the influence that prebiotics and probiotics, as well as supplemental proteins, have on the performance of Holstein calves is variable.

Milk replacer refusal was inversely proportional to age during the first 4 d of life, after which a refusal was rare and associated only with a morbid calf. There was no influence of treatment on the proportion of calves that refused milk replacer during the first 4 d of age (57 vs. 51% for control and prophylactic, respectively; *P* = 0.589). However, among calves that did refuse milk replacer, the prophylactic calves refused less milk replacer during that period (Table 2). Therefore, the prophylactic treatment lessened the intensity of milk refusal among calves that refused milk replacer. The underlying mechanism of the seemingly improved appetite among those calves that refused milk replacer during the first 4 d of life is unknown but could be attributed to less severe enteric disease.

Enteric morbidity, classified as having consecutive days with fecal scores ≥ 3 , was more common among control calves than among prophylactic calves (Table 3). These data are consistent with the literature. Inclusion of

prebiotics (Heinrichs et al., 2003), probiotics (Abe et al., 1995; Görgülü et al. 2003), and hyperimmunized egg protein (Ikemori et al., 1997; Hennig-Pauka et al., 2003) improved fecal scores and reduced scours. However, as with performance, data on the influences of prebiotics, probiotics, and hyperimmune proteins on enteric health are equivocal. Hill et al. (2008) did not observe any improvement in scours in calves less than 2 mo of age when milk replacer was supplemented with fructo-oligosaccharides. In addition, Cruywagen et al. (1996) did not observe improvements in the incidence of enteric morbidity when calves were supplemented with 5×10^7 cfu of *L. acidophilus*.

The age when scours were observed was not different between treatments (Table 3). Scours are a nonspecific symptom of enteric disease, and the etiology of spontaneous calf scours is complex and often involves more than one infectious agent, including various bacteria, viruses, and parasites. Because the clinical signs of scours are nonspecific, causative agents are difficult to ascertain without directly examining the fecal microbiota ecology. Age of the calf is usually the best diagnostic indicator, with *E. coli* being the major pathogen during the first week of life and rotavirus and cryptosporidiosis appearing in the calf etiology during the second and third

Table 3. Effect of prophylactic blend of prebiotics, probiotics, and hyperimmunized egg protein on the health of neonatal Holstein calves

Item	Treatment (Trt)			Main effect, <i>P</i> <		
	Control (n = 44)	Prophylactic (n = 45)	Largest SEM	Trt	Time	Trt × time
Enteric morbidity, ¹ %	51.1	25.0	—	0.011	—	—
Day of initiation of scours ²	12.0	10.0	1.45	0.264	—	—
Duration of scours, ² d	2.76	3.23	0.584	0.516	—	—
Total leukocyte counts, ×10 ⁶ /mL	7.77	7.89	0.276	0.762	0.428	0.470
Neutrophils, %	35.9	34.2	1.39	0.376	0.001	0.990
Neutrophil:mononuclear cells	0.638	0.625	0.0488	0.855	0.001	0.592
Hematocrit, %	31.2	30.6	0.59	0.506	0.001	0.309
Haptoglobin, ³ OD × 100	1.21	1.14	0.093	0.714	0.047	0.771
Plasma cortisol, ⁴ ng/mL	27.9	21.3	3.13	0.134	—	—
Plasma glucose, mg/dL	68.7	70.1	1.01	0.339	0.001	0.213
Plasma urea nitrogen, mg/dL	6.38	5.79	0.286	0.150	0.789	0.661

¹Significance reported from chi-squared probability test analysis. Classified as consecutive days with fecal scores ≥3.

²Data are reported only from calves that were classified as scouring (consecutive fecal scores ≥3).

³Significance reported from log₁₀ transformed data. OD = optical density.

⁴Cortisol was only analyzed on d-21 samples.

weeks of life (Blood, 2000). Future research should determine whether the prophylactic treatment used in the current study has different efficacies on different combinations of pathogens and ages of the calf.

Finally, the duration of scours among calves that developed scours was not different between treatments (Table 3). Control calves that scoured were treated with oral doses of antimicrobials, whereas scouring prophylactic calves were not treated with antimicrobials but were instead administered additional doses of the probiotics. It remains to be determined if the probiotics or additional probiotics during enteric disease improve recovery from the disease and, therefore, could be used as an alternative therapeutic to antimicrobials in the treatment of bacterial scours. In children, a meta-analysis on the influence of *Lactobacillus* spp. as a therapy in the treatment of acute diarrhea indicated a 0.7-d reduction in diarrhea duration as well as diarrhea frequency (Van Niel et al., 2002). However, not all probiotics may be equally effective. In children with acute diarrhea, 5 different probiotics were evaluated in addition to oral rehydration therapy,

and only one of the probiotics shortened the duration of diarrhea (Canani et al., 2007). Therefore, the proof of efficacy of each probiotic species or strain needs to be determined through research, and generalizations across strains or species should be limited to the development of hypotheses.

Treatment did not influence total peripheral blood leukocyte or differential counts (Table 3). In addition, there were no differences in hematocrit or plasma concentrations of haptoglobin, cortisol, glucose, or urea nitrogen (Table 3). These data indicate that the prophylactic treatment did not influence the systemic physiology of the calves during the first 21 d of life. The reduction in the proportion of peripheral leukocytes as neutrophils as well as the decreased plasma haptoglobin over time (data not shown) indicated either maturation of systemic physiology or adaptation of the calves to the ex utero environment. It cannot be determined whether the age-related changes in peripheral neutrophil counts or plasma haptoglobin concentrations were due to lower immunogenic stimulation or reduced stress.

Mortality rate in the current study was low; only one calf died (7-d-old control calf). In addition, only 2 calves (1 in each treatment) were classified as severely ill, laterally recumbent and completely anorectic (6 and 9 d old for control and prophylactic, respectively). Therapeutic treatment of severely ill calves included ceftiofur hydrochloride (Excenel, Pfizer Animal Health, New York, NY), flunixin meglumine (Banamine, Intervet/Schering-Plough), and subcutaneous lactated Ringer's solution. Therefore, the incidence of systemic infection was likely low. Thus, low calf morbidity rates in the present study did not allow for any potential benefits on systemic variables or mortality to be expressed. Future research should evaluate the potential of the prophylactic treatment used in the current study to reduce mortality on a group of high-risk, experimentally challenged, or immunocompromised (e.g., no colostrum) calves.

There are many plausible mechanisms explaining the reduced incidence of enteric morbidity when milk replacer fed to calves is supplemented with a combination of prebiotics, probiotics, and hyperimmune pro-

Table 4. Effect of prophylactic blend of prebiotics, probiotics, and hyperimmunized egg protein on functional innate immune responses of neonatal Holstein calves on d 21 of age

Item ¹	Treatment (Trt)		SEM	Trt, <i>P</i> <
	Control (n = 35)	Prophylactic (n = 35)		
Neutrophil L-selectin, GMFI	57.3	53.1	6.44	0.649
Neutrophil β_2 -integrin, GMFI	1,399.9	1,541.8	129.58	0.442
Neutrophil phagocytic/oxidative burst positive, %	70.4	70.7	2.17	0.917
Neutrophil phagocytic intensity, GMFI	27.8	26.5	3.26	0.784
Neutrophil oxidative burst intensity, GMFI	2,323	2,124	116.8	0.234
Whole blood culture tumor necrosis factor- α , pg/mL	1,099	1,199	159.8	0.661

¹GMFI = geometric mean fluorescence intensity.

teins. These include neutralization of potential pathogens (Bernet et al., 1994; Hennig-Pauka et al., 2003), direct bactericidal activity (Midolo et al., 1995), and immune modulation (Blum et al., 2002; Lomax and Calder, 2009). In the current study, there was no influence of treatment on the innate immune responses of peripheral leukocytes of Holstein calves when evaluated at 21 d of age (Table 4). These data agree with the findings of Letellier et al. (2001) where prebiotics and probiotics alone or in combination did not influence peripheral blood phagocyte function in neonatal piglets. However, the roles that prebiotics and probiotics play in immune modulation are inconclusive and are likely influenced by many environmental factors (Blum et al., 2002; Lomax and Calder, 2009). Regardless, the current data indicate that the benefits of a combined prebiotics, probiotics, and hyperimmune egg protein supplement on reducing enteric morbidity during the first 21 d of age are not likely associated with increases in nonspecific immune responses of Holstein calves.

IMPLICATIONS

A prophylactic combination of prebiotics, probiotics, and hyperimmune egg protein reduced the days with

spontaneous scours during the first 21 d of age when compared with treatment with a nonmedicated control milk replacer. There were no differences in performance or measures of systemic health due to the prophylactic treatment; however, morbidity and mortality rates were low in the current study, implying good general calf management. Future research should determine the efficacy of the current prophylactic treatment in calves that are at a higher risk of developing severe morbidity and subsequent mortality, such as in calves with unknown colostrum status or shipping-stressed calves.

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