J. Dairy Sci. 103:7351–7363
https://doi.org/10.3168/jds.2019-17480

Supplementing neonatal Jersey calves with a blend of probiotic bacteria improves the pathophysiological response to an oral *Salmonella enterica* serotype Typhimurium challenge

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ABSTRACT

The objectives of the current study were to determine the effects of supplementing a blend of probiotic bacteria (Provida Calf, MB Nutritional Sciences, Lubbock, TX) on the pathophysiological response to an oral *Salmonella enterica* serotype Typhimurium challenge in neonatal Jersey calves. Twenty-four Jersey bull calves within 24 h of birth were acquired from a local calf ranch, blocked by total serum protein and initial body weight, and randomly assigned to 1 of 3 treatments (n = 8). Calves were assigned to either (1) Control (CON); base milk replacer, (2) Control + *Salmonella* Typhimurium (CON+ST); base milk replacer and challenged with *Salmonella* Typhimurium on d 7; or (3) Provida Calf probiotics + *Salmonella* Typhimurium (PRO+ST); same milk replacer supplemented with a proprietary blend of *Lactobacillus casei* and *Enterococcus faecium* strains and challenged with *Salmonella* Typhimurium on d 7. The PRO+ST calves were supplemented for the first 3 d with 2×10^{10} cfu/d and then with 2×10^{9} cfu/d for the remainder of the study. The CON+ST and PRO+ST calves were each challenged with approximately 5×10^{6} cfu of *Salmonella* Typhimurium (ATCC# 14028), which was a mild challenge that did not cause scours in the calves. Peripheral blood samples were collected on d 0, 7, 10, 14, and 21 and analyzed for hematology; serum was collected and analyzed for haptoglobin, glucose, and urea N. Rectal temperatures were collected daily from d 6 to 21, when all calves were killed, so that persistent colonization of *Salmonella* Typhimurium and histomorphology of both the duodenum and ileum could be determined. Serum haptoglobin and urea N concentrations were increased among CON+ST on d 10. In contrast, the peak rectal temperature on d 10 in PRO+ST calves was 40.4°C, which was greater than that for CON and CON-ST (38.9°C and 39.7°C, respectively). The neutrophil percentage in peripheral circulation in PRO+ST calves was 55.4%, which was greater than that for CON and CON+ST (34.8 and 41.8%, respectively). Seven of the 8 PRO+ST calves had elevated neutrophil percentages on d 10 compared with d 7, whereas 4 of the 8 CON+ST calves had reduced neutrophil percentages on d 10 compared with d 7. Villus height-to-crypt depth ratios in the duodenum were greater among CON and PRO+ST calves, being 1.38, 0.84, and 1.43 for CON, CON+ST, and PRO+ST, respectively. In the ileum, the PRO+ST calves had greater villus height-to-crypt depth ratios than both the CON and CON+ST calves (1.64, 1.53, and 2.43 for CON, CON+ST, and PRO+ST, respectively). These data indicate that supplementing neonatal calves with the blend of probiotic bacteria used in the current study can influence the pathophysiological response to a mild enteric *Salmonella* Typhimurium challenge.
Key words: calf, health, probiotic

INTRODUCTION

Newborn dairy calves are extremely susceptible to gastrointestinal diseases. The mortality rate of preweaning dairy heifers reported in the most recent National Animal Health Monitoring System (**NAHMS**) reports ranges from 4.2 to 7.8%, with gastrointestinal disease being the primary reported disease (USDA NAHMS, 2007, 2011, 2014). Further, many calves with scours are treated with antimicrobials; USDA NAHMS (2011) reported that 71.8% of calves with scours were treated with antimicrobials. Supplementing probiotics to young calves could be an alternative therapeutic option for preventing scours and reducing the use of antimicrobials in neonatal calves. Timmerman et al. (2005) reported that calves supplemented with a blend of lactic acid–producing bacteria had reduced antibiotic use for gastrointestinal disease.

Supplementing probiotic bacteria has been reported to improve calf starter intake, growth, and various immune responses (Abe et al., 1995; Timmerman et al.,

Received August 20, 2019.
Accepted March 24, 2020.
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2005; Qadis et al., 2014). Supplementing probiotic bacteria may improve the resistance to enteric pathogens, especially while the gastrointestinal immune system of neonatal calves develops during the first few weeks of life (Zhao et al., 2003). Fleige et al. (2007) reported that dairy calves supplemented with *Enterococcus faecium* had greater villus height in the ileum compared with a control group. However, only Soto et al. (2016) investigated whether supplementing probiotic bacteria to dairy calves reduced the pathophysiological response following an oral LD_{50} (median lethal dose) challenge with *Salmonella* Dublin.

The objective of the current study was to determine whether supplementing a blend of 2 strains of lactic acid–producing bacteria with probiotic properties, *Lactobacillus casei* and *Enterococcus faecium*, can improve the immune responses, gastrointestinal tract development, and growth of neonatal Jersey bull calves following a mild oral *Salmonella* Typhimurium challenge.

MATERIALS AND METHODS

Calves and Treatments

All animal procedures were reviewed and approved by the Livestock Issues Research Unit (Lubbock, TX) of the USDA-Agricultural Research Service animal care committee. The study was conducted during April of 2016. Twenty-four Jersey bull calves (n = 8) were transported 300 km from a commercial calf ranch to the temperature-controlled calf barn at the Livestock Issues Research Unit. The calf barn was maintained at 21 ± 5.0°C. All calves were born within 24 h of the enrollment date (referred to as age 0 d). All calves were picked up from the calf ranch at 1000 h on a single day. A visual assessment of all eligible calves was conducted, and calves free of any congenital defects, with the ability to stand on their own when stimulated, and no visual signs of dehydration (no skin tenting or sunken eyes) were enrolled in the study. Upon arrival at the USDA calf barn, each calf was weighed on a Caf Cart Scale (Digi-Star, Fort Atkinson, WI) and a peripheral blood sample was collected by jugular venipuncture immediately into an evacuated tube without any additive. Serum was collected after the blood was allowed to clot for 30 min at 23°C and centrifuged at 1,200 × *g* for 20 min. Total serum protein (**TSP**) from each calf was recorded using a temperature-controlled handheld refractometer (Atago U.S.A. Inc., Bellevue, WA). Calves were first stratified by TSP, and 4 TSP blocks were created with 6 calves per TSP block. The mean TSP (±SD) of the 4 TSP blocks were 4.4, 5.4, 6.0, and 7.0 ± 0.08 g/dL. Subsequently, within each TSP block, calves were stratified by initial BW to create light and heavy initial BW blocks. The mean initial BW (±SD) of the light and heavy initial BW blocks were 24.9 and 29.5 ± 2.67 kg, respectively. The 3 calves in each TSP × initial BW block were then randomly assigned to 1 of 3 treatments by picking a corresponding treatment ear tag out of a bag without replacement. Therefore, 8 TSP × initial BW blocks were created with 3 calves in each block. Further, each block contained 1 calf from each of the 3 treatments. Calves were housed in individual pens (1.2 m × 2.1 m, width × length; Agri-Plastics, Tonawanda, NY) that prevented calf-to-calf physical contact. The 3 calves in each TSP × initial BW block were housed in pens next to each other. To prevent horizontal transmission of the *Salmonella* Typhimurium to the CON calves, any sample collected from calves was taken from the CON calves first, and all equipment, clothing, and boots were washed and disinfected before the next use. Further, their feed, milk bottles, and nipples were kept separate. No bedding was provided in the pens, as the floor was a calf mesh Tenderfoot (Tandem Products Inc., Minneapolis, MN) raised above a manure pit.

Calves were assigned to 1 of 3 treatments: (1) control (**CON**); base milk replacer, (2) control + *Salmonella* Typhimurium (**CON+ST**); base milk replacer and challenged with *Salmonella* Typhimurium on d 7; or (3) probiotics + *Salmonella* Typhimurium (**PRO+ST**); milk replacer supplemented with a proprietary blend of *Lactobacillus casei* and *Enterococcus faecium* strains (Provida Calf, MB Nutritional Sciences LLC, Lubbock, TX) and challenged with *Salmonella* Typhimurium on d 7. The PRO+ST calves were supplemented for the first 3 d with 2×10^{10} cfu/d of the probiotic and then with 2×10^{9} cfu/d for the remainder of the study. The logic behind supplementing with a 10-fold greater dose for the first 3 d of the study was to potentially displace any enteric bacterial pathogens that may have colonized between birth and when the calves were enrolled in the study. The PRO+ST treatment had the probiotic bacteria added directly to each bottle after mixing a single batch of milk replacer to ensure no variation in intake of milk solids among treatments. The total dose (cfu) supplemented per day was determined weekly by performing serial dilutions of the concentrated probiotic powder followed by spread plating 100 μL on de Man, Rogosa, and Sharpe agar (Sigma-Aldrich Inc., St. Louis, MO) and incubating at 37°C for 48 h in a BD GasPak EZ Anaerobe Pouch (BD, Franklin Lakes, NJ). Plates with 35 to 150 colonies per plate were counted to determine the actual dose (cfu) supplemented. The concentrated probiotic powder was stored at 4°C between feedings. All calves had ad libitum access to a common texturized calf starter (Ampli-Calf, Land O'Lakes Inc., Shoreview, MN). Calves were fed 500 g (DM) of milk solids per day, divided equally between 2

feedings at 0700 and 1600 h. The milk replacer was a 22% CP all-milk protein and 20% fat powder without any additional nutritional supplements added and was fed at 11.5% DM and 39 to 42°C.

Management of calves was identical among treatments. Fresh water, 8 L, was offered following the morning milk replacer feeding, and the amount remaining the following morning was determined to calculate the total voluntary water intake each day. Calf starter intake was recorded each morning by subtracting the orts from the previous day from the amount offered that day. The amount offered was adjusted daily to ensure approximately 20% orts. Calves were assessed for scours before and after each feeding, defined as a fecal consistency similar to that of pulpy orange juice. A 2-L bottle of an oral electrolyte (Electrolife Renew, MB Nutritional Sciences LLC, Lubbock, TX) was available for ad libitum consumption between each feeding, twice daily, until scouring ended.

Salmonella Typhimurium Challenge

The CON+ST and PRO+ST calves were each challenged with approximately 5×10^6 cfu of *Salmonella enterica* serotype Typhimurium that was resistant to nalidixic acid (ATCC# 14028). An individual colony from a streak plate was incubated overnight at 37°C in trypticase soy broth and agitated at 200 rpm. Then, 100 μL of the overnight culture was added to 20 mL of fresh trypticase soy broth and incubated at 37°C and 200 rpm until it reached an optical density of 0.8 at 450 nm. This was previously determined to be in the mid-logarithmic phase of growth. The broth was diluted to approximately 5×10^5 cfu/mL using sterile saline and each calf was given 10 mL of this solution added directly to the morning milk replacer. Calves were challenged within 30 min of creating the working bacteria solution. The CON calves were given 10 mL of sterile saline only. An estimate of the approximate challenge dose was determined by serially diluting the challenge dilution and spread plating on trypticase soy agar plates. Plates with 30 to 150 cfu were counted and the total challenge dose was estimated at 5.3×10^6 total cfu.

Observations and Sample Collection

Individual calf BW were recorded on d 0, 7, 14, and 21 on a Caf Cart Scale (Digi-Star), and the ADG was calculated as (BW at later measurement – BW at previous measurement)/days between measurements. Fresh fecal samples were collected directly from the rectum of each calf using digital stimulation on d 0, 7, 10, 12, 14, and 21 immediately following the morning milk replacer feeding. At least 5 g of fecal material was collected from each calf and stored in a Whirl-Pak bag (Nasco, Fort Atkinson, WI) at −80°C until analyzed for DM. Latex gloves were changed between calves. Rectal temperatures were collected each morning from 0600 to 0700 h from each calf on d 0 and from d 6 to 21 (daily) using a handheld thermometer (GLA Agricultural Electronics, San Luis Obispo, CA) that was disinfected with 70% ethanol between calves. Accuracy of the thermometer was confirmed each week by placing the thermometer in a water bath at various known temperatures between 38°C and 40°C. Peripheral blood samples were collected into 3-mL K_2EDTA and 10-mL no-additive Vacutainers (Becton Dickinson and Co., Franklin Lakes, NJ) on d 0, 7, 10, 14, and 21 by jugular venipuncture using a 21-gauge needle. The K_2EDTA vial was analyzed within 1 h for hematology on a Procyte Analyzer (Idexx Labs, Portland, ME) with bovine-specific algorithms. Data reported are total leukocyte counts and differentiation of lymphocytes, PMN, and monocytes; red blood cell (**RBC**) counts and morphology; hemoglobin concentrations; and hematocrit. Serum was collected from the no-additive Vacutainer after it was allowed to sit at 23°C for 30 min to clot, and then centrifuged at 1,500 × *g* for 20 min. Serum was stored at −80°C in quadruplicates until analyzed for serum glucose, urea N, and haptoglobin concentrations.

Fecal scores were assigned independently at each milk replacer feeding by 2 trained individuals on a 1 to 4 categorical scale, where 1 = firm, well-formed (not hard); 2 = soft, pudding-like; 3 = runny, pancake batter; 4 = liquid, splatters, pulpy orange juice (Larson et al., 1977). The individuals were not blinded to the treatments but, following each independent assessment, they compared their results and any disagreement in a calf's fecal score was reconciled by both observers through an additional assessment and discussion between the observers. Calves with 2 consecutive fecal scores of 4 were considered as having scours.

All calves that survived the 21-d observation period were humanely euthanized using a penetrating captive bolt followed immediately by exsanguination. Immediately, each calf was necropsied and samples of the duodenum, ileum, and mesenteric lymph nodes were collected. The duodenum and ileum samples were taken 10 cm distal to the pyloric-duodenal junction and 10 cm proximal to the ileocecal junction, respectively. The samples were cut longitudinally, rinsed with sterile PBS, and then added to a 50-mL conical tube filled with 10% formalin and stored until analyzed for histopathology. The third mesenteric lymph node from the ileocecal junction was collected and immediately transferred to the USDA-ARS Livestock Issues Research Unit to determine tissue colonization of the challenge *Salmonella*

Typhimurium. The gastrointestinal tract was dissected from each calf as well as the liver, spleen, and kidneys. The reticulorumen, abomasum, and omasum were rinsed free of any feed debris and each was weighed separately. Total weights of both the small and large intestines were recorded with digesta remaining. Last, liver, spleen, and kidney weights were recorded.

Sample Analysis

Fecal samples were weighed and dried at 90°C in a forced air oven for 48 h. The DM percentages of fecal samples were calculated as [(fecal sample dry weight + tray weight)/(fecal sample wet weight + tray weight) × 100]. Serum glucose and urea nitrogen concentrations were quantified in duplicate using a micro-manual method according to manufacturer's instructions (Stanbio Laboratory, Boerne, TX). Intra- and inter-assay coefficients of variation were 3.4 and 4.0% for serum glucose concentrations and 3.7 and 4.3% for serum urea nitrogen concentrations. Serum haptoglobin was measured in duplicate calorimetrically using the procedures outlined in Ballou et al. (2011). Data were converted from an optical density measurement to micrograms per milliliter by creating a regression equation using serum samples from cows with known haptoglobin concentrations ranging from 25 to 2,000 μg/mL. Intra- and inter-assay coefficients of variation were 4.2 and 5.5%, respectively, using a pooled serum sample from d 10 among challenged calves. Serum was pooled from d-10 challenged calves because they were expected to have elevated serum haptoglobin concentrations. The pooled sample was created from all challenged calves by pooling 250-μL aliquots from each calf.

Duodenum and ileum samples were trimmed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin in duplicate at the Texas Tech University Health Sciences Center Pathology Laboratory (Lubbock, TX). The slides were coded by the pathology laboratory so research staff were blinded to treatments when recording the histomorphological measurements. Pictures were taken using a Nikon Eclipse TS100 microscope with a Nikon ELWD 0.3 camera under 10× magnification (Nikon Instruments Inc., Tokyo, Japan). Nikon NIS elements F 2.20 (Nikon Instruments Inc.) software was used to quantify the average villus height, villus width, and crypt depth of 5 individual villi and crypts. The villus height-to-crypt depth ratio was calculated by measuring a villus height and the crypt depth under the villus.

Mesenteric lymph nodes were submerged for 5 s in boiling water to sterilize the surface. A 1-g sample was aseptically dissected and transferred to a sterile bag with 100 mL of sterile PBS and then homogenized using a Seward stomacher (Seward Ltd., Worthing, UK) for 2 min at 230 rpm. One hundred microliters of the homogenate was plated directly on CHROMagar Salmonella Plus agar (CHROMagar, Paris, France). Additionally, each homogenate sample was enriched for *Salmonella* spp. in tetrathionate broth overnight, and 100 μL was plated directly on CHROMagar Salmonella Plus and selective XLD (Sigma-Aldrich, St. Louis, MO) agar with nalidixic acid (Sigma-Aldrich) at 50 μg/mL to confirm that the *Salmonella* sp. was likely the challenge microorganism. The Petri dishes were enumerated by trained research staff who were blinded to treatments.

Statistical Analyses

Sample size estimation was calculated using 1-sided means comparisons for 2 variables, rectal temperature and total peripheral blood neutrophil counts, and 5% and 20% protections against type 1 and type 2 errors, respectively. The estimated effect size (±sample SD) was 40.0 vs 40.5 (±0.3°C) for rectal temperature and 3.0 vs 4.5 (±1.0 × 10^6 cells/mL) after the *Salmonella* Typhimurium challenge. Four calves died during the study. Data from those 4 calves were included in the statistical analyses until they died.

All continuous, repeated data were analyzed by REML ANOVA using the MIXED procedure of SAS (v.9.4, SAS Inst. Inc., Cary, NC). The model included the fixed effects of treatment, time, and treatment × time, and the random effect of block. The subject of the repeated statement was calf nested within treatment, and the appropriate covariance structure for the within-calf measurements was chosen based on the lowest Bayesian information criterion. All continuous, nonrepeated data were analyzed by REML ANOVA using the MIXED procedure of SAS (v.9.4) with the fixed effect of treatment and random effect of block. Normality of the residuals was evaluated using the Shapiro-Wilk statistic and normal probability plots using the UNIVARIATE procedure of SAS (v.9.4). Pairwise differences were assessed at each time interval using a sliced effect multiple comparison approach with a Tukey-Kramer adjustment. Before statistical analyses, fecal score data were averaged by week and analyzed as a repeated measure using the REML ANOVA described above for continuous, repeated data using the MIXED procedure of SAS (v.9.4). Further, the proportion of calves treated for scours both before and after the *Salmonella* Typhimurium period was analyzed using a Fisher's exact test using the FREQ procedure of SAS (v.9.4). Additional analyses were performed on rectal temperatures and neutrophil counts in peripheral blood. Simple bivariate linear regressions were performed using either rectal temperature or neutrophil

count on d 10 as the response variable and TSP at enrollment as the predictor variable, using only the CON+ST and PRO+ST calves. These were performed using the REG procedure of SAS (v.9.4). Differences of $P \leq 0.05$ were considered significant, and $0.10 \geq P > 0.05$ was considered a tendency.

RESULTS

Performance, Plasma Metabolites, and Health

Four calves died during the study. Two CON calves died, the first on d 7 and the second on d 8. One calf died in each of the PRO+ST and CON+ST treatments, on d 12 and 13, respectively. Both of the *Salmonella* Typhimurium-challenged calves were in the lowest TSP block. All 4 calves died due to suspected septicemia; however, definitive causes of death could not be determined because necropsies were not performed. The ADG, starter intake, water intake, serum metabolites and haptoglobin concentrations, and fecal characteristics are reported in Table 1. Although no treatment × time interaction was detected ($P = 0.360$), the CON+ST and PRO+ST calves tended to have greater serum glucose concentrations than CON calves ($P = 0.071$). There was a treatment × time interaction for serum urea nitrogen concentration ($P = 0.013$). Serum urea nitrogen concentration by time is reported in Figure 1, and it indicated that the CON+ST had elevated serum urea nitrogen on d 10 ($P \leq 0.038$) compared with both CON and PRO+ST. In addition, there was an interaction of treatment × time ($P = 0.015$) on serum haptoglobin concentration, which is reported in Figure 2. Those data illustrate that CON had a greater serum haptoglobin concentration than the other 2 treatments on d 7 ($P \leq 0.015$); however, CON+ST had elevated serum haptoglobin concentration on d 10 ($P = 0.035$) compared with PRO+ST calves, whereas there was no difference between CON and PRO+ST ($P = 0.279$). Moreover, CON calves had greater serum haptoglobin concentration than either CON+ST or PRO+ST on d 21 ($P \leq 0.029$). Last, we detected an interaction of treatment × time ($P = 0.004$) on rectal temperature, which is shown in Figure 3. The CON+ST and PRO+ST calves had increased rectal temperature on d 9 ($P \leq 0.011$). The PRO+ST calves had the greatest rectal temperature ($P \leq 0.004$) and CON+ST had increased rectal temperature compared with CON ($P = 0.005$) on d 10. In addition, rectal temperature was greater in PRO+ST than CON on d 11 ($P = 0.003$). There was a tendency ($P = 0.065$; $R^2 = 0.223$) in the simple linear regression of rectal temperature at d 10 × TSP at enrollment, with a positive slope estimate of 0.419.

Hematology Analysis

Hematology data are reported in Table 2. No interaction of treatment × time was observed in total leukocyte counts ($P = 0.465$); however, there was a tendency for a treatment difference ($P = 0.074$). Moreover, there was no interaction of treatment × time in PMN counts ($P = 0.135$); in contrast, there was a difference among treatments for PMN counts ($P = 0.035$). We found no interaction of treatment × time in monocyte counts ($P = 0.128$), but there was a tendency for a treatment

Table 1. Growth performance, intakes, concentrations of serum metabolites and haptoglobin, fecal characteristics, and rectal temperature of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella enterica* Typhimurium[1]

Variable	Treatment (Trt)			Largest SEM	*P*-value		
	CON	CON+ST	PRO+ST		Trt	Time	Trt × time
ADG, kg/d	0.153	0.163	0.178	0.0348	0.846	0.001	0.577
Starter intake, kg/d	0.028	0.044	0.027	0.0149	0.651	0.001	0.999
Water intake, L/d	0.57	0.59	0.53	0.114	0.929	0.001	0.277
Serum glucose, mg/dL	86.6	95.9	95.1	2.98	0.071	0.001	0.360
Serum urea N, mg/dL	8.5	11.0	10.1	1.28	0.385	0.001	0.013
Serum haptoglobin, μg/mL	350	268	176	48.5	0.045	0.005	0.015
Fecal score,[2] weekly mean	2.60	2.93	2.82	0.294	0.689	0.045	0.521
Pre-challenge scours,[2] % of calves	75	63	50	—	0.855	—	—
Post-challenge scours,[2] % of calves	50	57	57	—	0.949	—	—
Fecal DM, %	19.5	21.7	23.4	1.73	0.247	0.001	0.499
Rectal temperature, °C	38.8	38.8	39.0	0.10	0.078	0.001	0.004

[1]Treatments included an unsupplemented control (CON; n = 6), an unsupplemented control challenged with *Salmonella* Typhimurium on d 7 (CON+ST; n = 7), and a probiotic-supplemented group that was also challenged with the *Salmonella* Typhimurium on d 7 (PRO+ST; n = 7). Calves were studied from d 1 to 21 of life.

[2]Fecal scores were determined twice daily before and after each milk replacer feeding. Feces were classified on a 1 to 4 categorical scale, where 1 = firm, well-formed (not hard); 2 = soft, pudding-like; 3 = runny, pancake batter; 4 = liquid, splatters, pulpy orange juice (Larson et al., 1977). A calf with 2 consecutive fecal scores of 4 was considered to have scours.

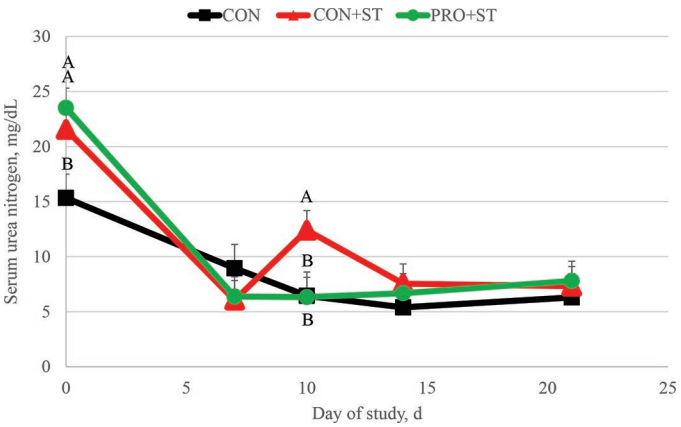

Figure 1. Serum urea N concentrations (mean ± SEM) of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.013$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A, B) for each sample time.

difference ($P = 0.081$). We detected an interaction of treatment × time in the percentage of PMN ($P = 0.028$; Figure 4); PRO+ST had an increased percentage of PMN on d 10 ($P \leq 0.050$) compared with either CON or CON+ST. There was a tendency ($P = 0.099$; $r^2 = 0.195$) in the simple linear regression of neutrophil counts in peripheral blood at d 10 × TSP at enrollment, with a positive slope estimate of 1.153. Additionally, we detected an interaction of treatment × time in the percentage of lymphocytes ($P = 0.027$; Figure 5); on d 10, PRO+ST had a reduced percentage of lymphocytes compared with CON ($P = 0.007$). The percentage of monocytes also had a treatment × time interaction ($P = 0.033$; Figure 6), whereas PRO+ST had a greater percentage of monocytes on d 14 ($P \leq 0.022$). Finally, there was a treatment × time interaction in the ratio of PMN to lymphocyte counts ($P = 0.024$; Figure 7): PRO+ST had the greatest PMN to lymphocyte ratio on d 10 ($P \leq 0.030$).

Small Intestinal Morphology and Salmonella enterica Colonization

Duodenum and ileum histomorphology data are reported in Table 3. We found no treatment differences in either villus height or crypt depth in the duodenum ($P \geq 0.125$); however, there was a tendency for a treatment difference ($P = 0.075$) in the ratio of villus height to crypt depth. Both PRO+ST and CON tended to have greater ratios of villus height to crypt depth in the duodenum compared with CON+ST ($P \leq 0.054$). There were no treatment differences in the villus height of the ileum ($P = 0.542$). However, PRO+ST had decreased crypt depth compared with either CON or CON+ST ($P = 0.005$). In addition, the villus height-to-crypt ratio in the ileum was greater in PRO+ST than in CON or CON+ST ($P = 0.001$). Finally, we found no treatment differences in the proportion of calves that were colonized in the mesenteric lymph nodes with the challenge strain of *Salmonella enterica* ($P = 0.263$).

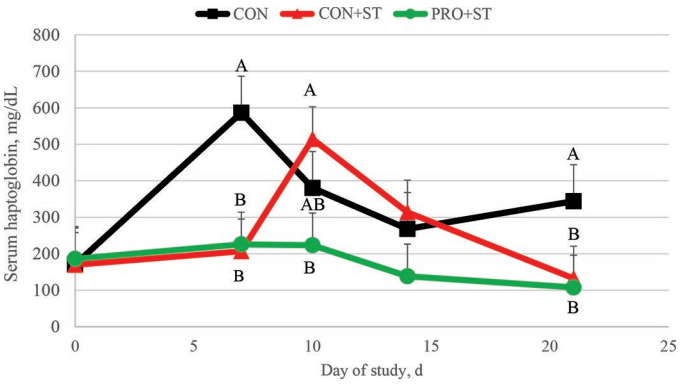

Figure 2. Serum haptoglobin concentrations (mean ± SEM) of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.015$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A, B) for each sample time.

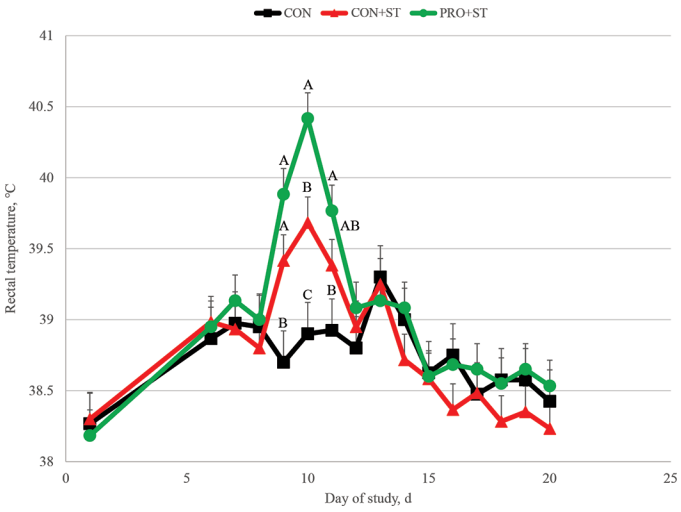

Figure 3. Rectal temperatures (mean ± SEM) of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.004$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A–C) for each sample time.

None of the CON were colonized with the challenge strain of the *Salmonella enterica*.

Organ Weights

The absolute weights of the stomach, liver, intestines, spleen, kidneys, as well as those organ weights expressed relative to final BW, are reported in Table 4. There were no treatment differences ($P \geq 0.181$) in any of the organ weights or organ weights relative to final BW.

DISCUSSION

This study investigated the growth performance, immune responses, and gastrointestinal tract health and histomorphology of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium. The current data suggested that the probiotic supplement improved intestinal health and reduced systemic inflammation following a moderate infectious dose of *Salmonella* Typhimurium.

The effects of supplementing probiotic bacteria on the performance of dairy calves is equivocal. Timmerman et al. (2005) reported greater ADG of preweaning calves supplemented with a combination of 5 different strains of *Lactobacillus* spp. and one *Enterococcus faecium* strain at a dose of 1×10^9 cfu/kg of BW. In contrast, Ballou (2011) reported no difference in either starter intake or ADG among a group of 45 calves that were supplemented with a blend of prebiotics, probiotic bacteria, and egg protein from laying hens vaccinated against various enteric pathogens compared with a group of 45 negative control calves. Among enteric-challenged animals, Cao et al. (2013) reported that chickens supplemented with *Enterococcus faecium* had greater ADG than negative controls after an oral *Escherichia coli* K88 challenge. In contrast, there was no difference in BW gain between *Enterococcus faecium*-supplemented pigs and negative control pigs after oral challenge with *Salmonella* Typhimurium (Szabó et al., 2009). The lack of an effect on either ADG or BW gain in the current study could be attributed to the short observation period of only 21 d, which also corresponds with a period when calves are not consuming a lot of calf starter.

There were no differences in either fecal score, incidence of scours, or DM content of feces among treatments. Further, the challenge *Salmonella* Typhimurium used in the current study did not increase fecal scores or incidence of scours or reduce fecal DM because neither the CON+ST nor PRO+ST fecal characteristics were different from those of the unchallenged CON calves. The lack of a *Salmonella* Typhimurium effect on fecal consistency could be masked by the high incidence of spontaneous scours seen in calves at this age. Ballou (2011) reported that newborn Holstein calves supplemented with a blend of prebiotics, probiotics, and immunized egg protein did not differ in the day that scours initiated or in the duration of scours; however, they reported that fewer supplemented calves had a fecal score >3. Timmerman et al. (2005) reported similar results, where preweaning calves supplemented a probiotic blend containing 5 *Lactobacillus* spp. and an

Table 2. Hematology of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella enterica* Typhimurium[1]

Variable	Treatment (Trt)			Largest SEM	*P*-value		
	CON	CON+ST	PRO+ST		Trt	Time	Trt × time
Red blood cells, $\times 10^6/\mu L$	8.14	7.32	8.64	0.611	0.326	0.245	0.921
Hemoglobin, g/dL	10.12	9.33	10.63	0.755	0.469	0.905	0.869
Hematocrit, %	32.8	29.8	34.1	2.90	0.513	0.909	0.811
Mean corpuscular volume, fL	39.1	39.3	41.1	0.95	0.126	0.001	0.431
Mean corpuscular hemoglobin, pg	30.9	31.9	31.2	0.46	0.238	0.002	0.325
Platelet, $\times 10^6$/mL	591	557	490	50.8	0.259	0.189	0.233
Total leukocyte count, $\times 10^6$/mL	8.96	10.82	9.68	0.719	0.074	0.130	0.465
Neutrophil, $\times 10^6$/mL	2.85[a]	4.16[b]	3.95[ab]	0.439	0.035	0.001	0.135
Lymphocyte, $\times 10^6$/mL	5.39	5.54	4.88	0.424	0.474	0.032	0.536
Monocyte, $\times 10^6$/mL	0.44	0.79	0.86	0.136	0.081	0.002	0.128
Eosinophil, $\times 10^3$/mL	17.5	9.0	8.8	7.45	0.618	0.039	0.405
Neutrophil, %	35.8	34.3	38.4	3.77	0.607	0.001	0.028
Lymphocyte, %	57.1	55.6	52.6	3.69	0.704	0.001	0.027
Monocyte, %	5.0	8.1	9.1	1.38	0.113	0.001	0.033
Eosinophil, %	0.16	0.10	0.09	0.076	0.747	0.034	0.409
Neutrophil:lymphocyte ratio	0.67	0.76	0.81	0.156	0.851	0.001	0.024

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

[1]Treatments included an unsupplemented control (CON; n = 6), an unsupplemented control challenged with *Salmonella* Typhimurium on d 7 (CON+ST; n = 7), and a probiotic-supplemented group that was also challenged with the *Salmonella* Typhimurium on d 7 (PRO+ST; n = 7). Calves were studies from d 1 to 21 of life.

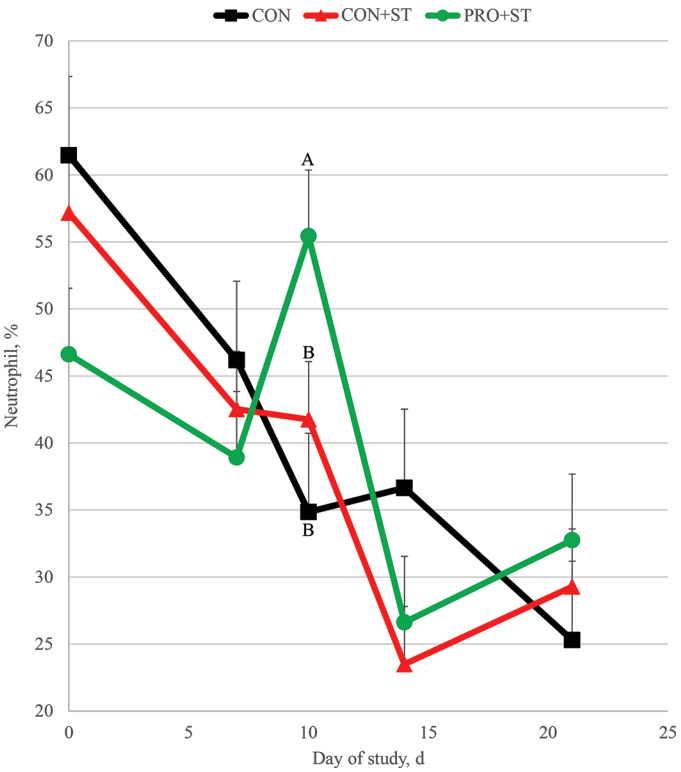

Figure 4. The neutrophil percentage (mean ± SEM) in blood of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.028$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A, B) for each sample time.

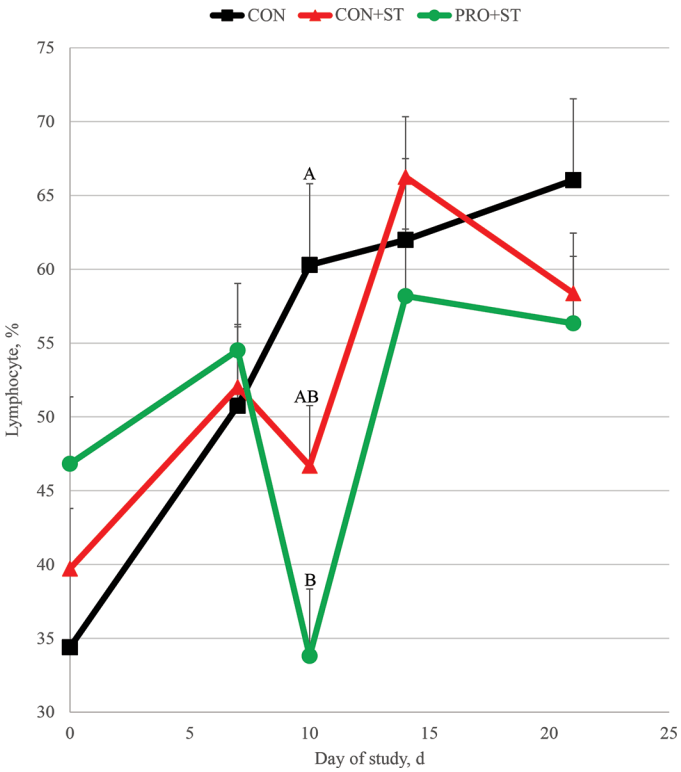

Figure 5. The lymphocyte percentage (mean ± SEM) in blood of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.027$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A, B) for each sample time.

Enterococcus faecium had a reduced incidence of scours compared with unsupplemented calves. Additionally, Abe et al. (1995) observed that newborn calves that received *Lactobacillus pseudolongum* or *Lactobacillus acidophilus* had reduced incidence of scours compared with a negative control group of calves.

Serum urea nitrogen concentration was elevated on d 10 among the CON+ST calves. Elevated serum urea nitrogen concentrations are consistent with results of Santos et al. (2002b), who reported that 3- to 4-wk-old calves challenged with *Salmonella enterica* had increased plasma urea nitrogen compared with a group of unchallenged calves. Further, the serum urea nitrogen data indicate that the CON+ST group had increased protein catabolism after the *Salmonella* Typhimurium challenge compared with either the CON or PRO+ST group, which is likely associated with a stronger acute phase response (Ballou et al., 2008). In contrast, 28 Holstein calves supplemented with a probiotic inoculum composed of a blend of *Lactobacillus casei*, *Lactobacillus salivarius*, and *Pediococcus acidilactici* and challenged with *Salmonella* Dublin did not have any difference in plasma urea nitrogen compared with a negative control group not supplemented with the probiotic blend (Soto et al., 2016).

Serum haptoglobin was also greater in CON+ST compared with PRO+ST on d 10, which further supports that the CON+ST had a greater acute phase response on d 10. Moreover, serum haptoglobin concentration is a reliable indicator of systemic inflammation, especially during *Salmonella enterica*-induced infection in calves (Deignan et al., 2000). Yin et al. (2014) conducted a study where 16 weaned pigs supplemented with *Lactobacillus casei* had reduced plasma haptoglobin concentrations compared with control pigs after a *Salmonella* Typhimurium challenge. In another study, a group of 16 weaned pigs that were supplemented with *Enterococcus faecium* tended to have decreased plasma haptoglobin concentrations compared with control pigs after a *Salmonella* Typhimurium challenge (Kreuzer et al., 2012). These data further support that the blend of *Lactobacillus casei* and *Enterococcus faecium* strains

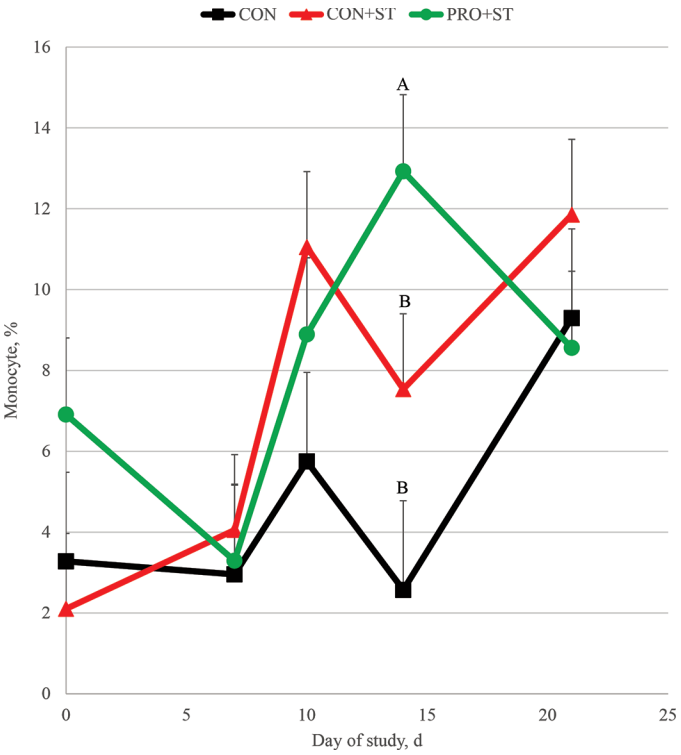

Figure 6. The monocyte percentage (mean ± SEM) in blood of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.033$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A, B) for each sample time.

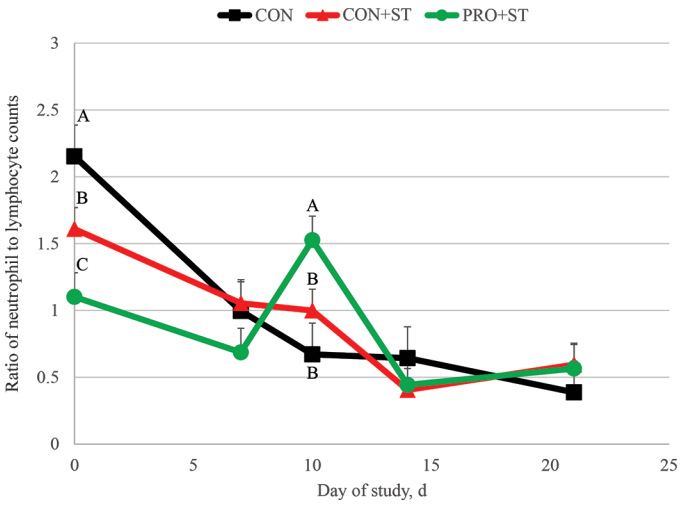

Figure 7. The ratio of neutrophil to lymphocyte (mean ± SEM) counts in blood of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium (PRO+ST) on d 7. Additional control treatments included an unsupplemented control (CON) and an unsupplemented control that was challenged with the *Salmonella* Typhimurium (CON+ST). There was a treatment × time ($P = 0.024$) interaction. Tukey-adjusted $P \leq 0.05$ pairwise differences are shown with differing letters (A–C) for each sample time.

used in the current study can reduce systemic inflammation in neonatal calves.

Rectal temperatures peaked at an average of 40.4°C among PRO+ST calves, which was higher than the average of either the CON or CON+ST calves. The serum haptoglobin data and rectal temperature in the current study may initially seem contradictory because the CON+ST calves had greater serum haptoglobin, but the PRO+ST calves had greater rectal temperatures. However, the relationship between different measurements of infection and inflammation may not be that straightforward. Smith et al. (1998) did not observe a correlation between serum haptoglobin concentrations and rectal temperature in dairy cattle with toxic puerperal metritis. However, others reported that plasma haptoglobin concentrations were positively correlated with rectal temperature within 3 d after a bacterial challenge (Hall et al., 1992; Godson et al., 1996; Deignan et al., 2000; Petersen et al., 2002). The low TSP block in this study had the most severe response

Table 3. Histomorphology of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella* Typhimurium[1]

Variable	Treatment			Largest SEM	Treatment *P*-value
	CON	CON+ST	PRO+ST		
Duodenum					
Villus height, μm	430	283	376	65.8	0.202
Crypt depth, μm	311	340	270	24.3	0.125
Villus height:crypt depth	1.38	0.84	1.43	0.155	0.075
Ileum					
Villus height, μm	455	421	483	38.5	0.542
Crypt depth, μm	276[a]	293[a]	202[b]	16.5	0.005
Villus height:crypt depth	1.64[a]	1.53[a]	2.43[b]	0.103	0.001
Mesenteric lymph node colonization, %	0	42.9	14.3	—	0.263

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

[1]Treatments included an unsupplemented control (CON; n = 6), an unsupplemented control challenged with *Salmonella* Typhimurium on d 7 (CON+ST; n = 7), and a probiotic-supplemented group that was also challenged with the *Salmonella* Typhimurium on d 7 (PRO+ST; n = 7).

Table 4. Tissue weights of Jersey calves supplemented with a blend of probiotic bacteria and challenged with *Salmonella enterica* Typhimurium[1]

Variable	Treatment			Largest SEM	Treatment *P*-value
	CON	CON+ST	PRO+ST		
Final BW, kg	28.7	30.6	28.6	2.01	0.611
Total stomach weight, kg	0.712	0.853	0.730	0.0964	0.347
Total stomach weight, % of BW	2.35	2.67	2.44	0.283	0.607
Total intestinal weight, kg	1.600	1.808	1.671	0.1200	0.398
Total intestinal weight, % of BW	5.46	5.67	5.60	0.475	0.943
Liver weight, kg	0.767	0.733	0.700	0.0552	0.631
Liver weight, % of BW	2.49	2.31	2.34	0.114	0.454
Spleen weight, kg	0.105	0.119	0.117	0.0095	0.464
Spleen weight, % of BW	0.34	0.37	0.40	0.032	0.488
Kidney weight, kg	0.289	0.229	0.255	0.0316	0.269
Kidney weight, % of BW	0.94	0.72	0.85	0.096	0.181

[1]Treatments included an unsupplemented control (CON), an unsupplemented control challenged with *Salmonella* Typhimurium on d 7 (CON+ST), and a probiotic-supplemented group that was also challenged with the *Salmonella* Typhimurium on d 7 (PRO+ST). Calves were studied from d 1 to 21 of life.

to the *Salmonella* Typhimurium challenge, irrespective of treatment; indeed, the only 2 calves that died after the challenge were in the lowest TSP block. Among *Salmonella* Typhimurium-challenged calves, there was a mild correlation among TSP and peak rectal temperatures, irrespective of treatment. These data further support that the elevated rectal temperature among the PRO+ST calves may actually reflect a less severe acute phase response. Further supporting elevated rectal temperatures among probiotic-treated animals, Szabó et al. (2009) observed that 69% of weaning pigs supplemented with *Enterococcus faecium* had a rectal temperature greater than 40.4°C, whereas only 39% of the control group of piglets had rectal temperatures over 40.4°C. These data illustrate that the effects of probiotic bacteria on the resistance and response to enteric infections is complex, and interpretation of any single variable must be made in the context of other variables of infection and disease that were measured.

We found no differences in RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentrations, or platelet counts. However, Santos et al. (2002b) reported that 3- to 4-wk-old Holstein calves challenged with *Salmonella* Typhimurium had greater RBC and hemoglobin after the challenge than before the challenge. Moreover, reduced RBC and hemoglobin were observed in rabbits supplemented with approximately 1.25×10^6 cfu/d of *Lactobacillus plantarum* compared with a control group (Bovera et al., 2012). Although we detected no difference in total leukocyte counts among treatments, calves challenged with *Salmonella* Typhimurium had greater PMN counts on d 10 and 21. Further, the PRO+ST group had increased PMN percentage on d 10 compared with d 7. In contrast, the PMN percentage in CON+ST did not change before and after the challenge. Seven of the 8 PRO+ST calves had elevated PMN on d 10 compared with d 7, whereas 4 of the 8 CON+ST calves had decreased PMN on d 10 relative to d 7, which resulted in greater PMN percentage in the PRO+ST group than in the CON+ST group on d 10. Infiltration of PMN into the infection site is likely the major reason for the decreased PMN percentage in blood circulation after the *Salmonella* Typhimurium challenge. A study conducted with 4- to 5-wk-old Holstein calves where ileal ligated loops were inoculated with 7.5×10^8 cfu of the same *Salmonella* Typhimurium used in the current study reported PMN recruitment into the mucosal tissue 12 h after *Salmonella* Typhimurium inoculation (Santos et al., 2002a). In addition, a study with broilers reported that chickens supplemented with *Enterococcus faecium* had greater heterophil count than chickens in the negative control treatment 3 d post-infection with a *Salmonella* Typhimurium (Levkut et al., 2012). Nightingale et al. (2015) reported that periparturient cows with comparatively high plasma haptoglobin concentrations had decreased PMN counts in peripheral blood circulation. Moreover, Malago et al. (2010) demonstrated that *Lactobacillus casei* can suppress IL-8 production, which may decrease PMN recruitment to the intestines. Further, an in vitro study demonstrated that an *Enterococcus faecium* supplement can decrease IL-8 protein expression in Caco-2 and IPEC-J2 cells after an *E. coli* challenge (Klingspor et al., 2015). Similar to our rectal temperature data, there was a mild positive correlation between TSP and PMN counts in peripheral circulation on d 10. These data suggest that PRO+ST can either prevent *Salmonella* Typhimurium from infecting the epithelium of the small intestine or decrease excessive recruitment of neutrophils to the intestines, or both.

A decrease in peripheral circulating lymphocytes is common during inflammation and was observed among

calves on d 10 that were challenged with *Salmonella* Typhimurium. Lymphocytes in peripheral circulation, on a percentage basis, were decreased to a greater extent among the PRO+ST calves. This difference on a percentage basis could be explained in part by the greater PMN counts among the PRO+ST, as described in the previous paragraph. The decreased lymphocytes in the peripheral circulation during inflammation is likely attributed to increased recirculation of these cells within the lymph to increase antigen specific recognition. Although effector lymphocyte responses were not quantified in the current study, Rieger et al. (2015) reported that pigs supplemented with *Enterococcus faecium* had greater effector intraepithelial lymphocytes in small intestinal mucosa on d 28 post-infection of *Salmonella* Typhimurium. The role that this combination of probiotic bacteria or other probiotics plays in development of mucosal adaptive immune responses should be investigated in future studies.

Monocytes are stimulated by IFN-γ, which is released by T-helper 1 cells after an intracellular pathogen challenge. Furthermore, Charleston et al. (2002) reported that six 30-d-old calves that were inoculated with 5×10^6 plaque-forming units of bovine viral diarrhea virus had elevated IFN-γ production on d 7 post-infection. Similar results were observed in the current study where monocyte counts increased on d 14 in *Salmonella* Typhimurium-challenged calves. Additionally, the PRO+ST calves had a greater percentage of monocytes than calves in the other 2 treatments on d 14. This observation was supported by Shida et al. (2006), who observed that a *Lactobacillus casei* strain could be phagocytosed by human mononuclear cells and then induced the production of IFN-γ and IL-12. Those authors also reported that natural killer cell production increased due to IL-12 released from monocytes.

No study has previously investigated the effects of *Lactobacillus casei* and *Enterococcus faecium* on the histomorphology of the small intestine of preweaning calves after a *Salmonella* Typhimurium challenge. However, the positive effects of *Lactobacillus casei* and *Enterococcus faecium* on the small intestinal morphology of mice, poultry, and pigs after an enteric challenge have been demonstrated previously (Aliakbarpour et al., 2012; Shukla et al., 2013; Rieger et al., 2015; Liu et al., 2017). Changes in villus height and crypt depth are related to the health of villus enterocytes and stem cell proliferation in the crypt, respectively. Therefore, the lower villus height-to-crypt depth ratio observed among CON+ST calves may have resulted from the mucosal invasion by *Salmonella* Typhimurium, which can lead to enterocyte damage and stimulate proliferation of stem cells in the crypts (Nabuurs et al., 1993). Accordingly, the villus height-to-crypt depth ratio is an indicator of a healthy small intestine. Therefore, data from the current study suggested that the PRO+ST had greater intestinal health than either the CON or CON+ST. Shukla et al. (2013) reported that, compared with the control treatment, mice supplemented with *Lactobacillus casei* had improved micro-villi morphology in the small intestine after *Giardia intestinalis* trophozoites challenge. Additionally, greater villus height and less crypt depth in jejunum sections were observed in the small intestines of broilers supplemented with *Enterococcus faecium* (Cao et al., 2013).

The colonization of *Salmonella* Typhimurium in the mesenteric lymph nodes reflects the invasiveness or persistence of the pathogen. de Waard et al. (2002) reported that rats supplemented with *Lactobacillus casei* had no difference in *Listeria monocytogenes* colonization than controls after a challenge. Moreover, no difference was observed in the colonization of mesenteric lymph nodes between pigs supplemented, or not, with *Enterococcus faecium* or not after a *Salmonella* Typhimurium challenge (Szabó et al., 2009; Kreuzer et al., 2012).

Infection of *Salmonella* Typhimurium in animals can induce local inflammatory responses, especially in the small intestinal mucosa. Additionally, severe infections can cause systemic inflammation, which can influence the normal function of organs. Thus, the weights of certain tissues can change during and immediately after a diseased state. Although other indicators of systemic inflammation after the *Salmonella* Typhimurium challenge indicated that the PRO+ST had a reduced inflammatory response compared with CON+ST, there were no differences in the weights of liver. In agreement, a study with mice demonstrated no difference in liver weight between a *Lactobacillus casei*-supplemented group and a control group after a *Salmonella* Typhimurium challenge (Jain et al., 2008). Moreover, de Waard et al. (2002) illustrated that rats supplemented with *Lactobacillus casei* had no difference in either liver or spleen weight compared with control rats after a *Listeria monocytogenes* challenge. In contrast, a poultry study suggested that chickens supplemented with a blend of *Enterococcus faecium*, prebiotic, and immune-modulating substances had reduced liver and spleen weights compared with other treatments; however, those chickens were not challenged with any specific pathogens (Awad et al., 2009).

CONCLUSIONS

A probiotic blend of *Lactobacillus casei* and *Enterococcus faecium* supplemented to neonatal Jersey calves appeared to modulate both local and systemic immune responses following a mild oral *Salmonella* Typhimurium challenge at 7 d of age. Supplementing the blend

of probiotics to these neonatal Jersey calves reduced systemic production of haptoglobin and serum urea nitrogen, increased rectal temperature, and improved histomorphology of the duodenum and ileum. Further data are needed to identify the specific mechanisms of this improved immune response.

ACKNOWLEDGMENTS

The authors thank Jeff Dailey and Rand Broadway from the USDA-ARS Livestock Issues Research Unit (Lubbock, TX) for their help with facility maintenance and microbiology of the mesenteric lymph nodes, respectively. M. A. Ballou has equity ownership in MB Nutritional Science. M. A. Ballou was involved in the design of the experiment, consulted on data analyses, and edited the final version of the manuscript. Y. Liang is now employed by MB Nutritional Sciences, but was not when the study was conducted, analyzed, and manuscript prepared. R. E. Hudson does not have any conflict of interest.

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