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Exploratory cohort study to determine if dry cow vaccination with a *Salmonella* Newport bacterin can protect dairy calves against oral *Salmonella* challenge

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Abstract

Background: Salmonellosis is a major cause of morbidity and mortality in neonatal calves, often occurring before preventative vaccines can be administered.

Hypothesis/Objective: To evaluate the protective effect on calves of colostrum from cows vaccinated with a commercially available *Salmonella* Newport bacterin against a *Salmonella* Typhimurium challenge.

Animals: Twenty Holstein bull calves from a university dairy farm.

Methods: Nonrandomized placebo-controlled trial in which colostrum was harvested from 30 cows that received 2 doses of either *Salmonella* bacterin or saline before calving. Colostrum collected from each group was pooled and fed to 2 groups of 10 calves at birth. At approximately 2 weeks of age, calves were challenged with *Salmonella* Typhimurium. Clinical, hematologic, microbiological, and postmortem findings were compared between the 2 groups.

Results: No differences in mortality, clinical findings, hematology results, blood and fecal cultures, or necropsy findings between the 2 groups were observed. Vaccinated cows had higher colostral titers, and calves fed this colostrum had higher serum titers (mean difference, 0.429; mean [SE], 0.852 [0.02] for vaccinated versus 0.423 [0.02] for control calves).

Conclusions and Clinical Importance: Transfer of colostral immunoglobulins from *Salmonella enterica* serotype Newport bacterin to neonatal calves was not sufficient to decrease mortality, clinical signs, sepsis, intestinal damage, or fecal shedding when exposed to a highly pathogenic *Salmonella* isolate. A large-scale randomized controlled clinical trial is needed to evaluate the efficacy of this bacterin when administered in the dry period for prevention of salmonellosis in neonatal calves.

KEYWORDS
calves, colostrum, passive immunity, *Salmonella*

Abbreviations: CTL, control group; IgG, immunoglobulin G; IHC, immunohistochemistry; OD, optical density; PD, potential difference; PP, plasma protein; SRP, siderophore-receptor and porin; TER, transepithelial electrical resistance; VAX, vaccinated group.

1 | INTRODUCTION

Diarrhea remains the most common cause of morbidity and mortality in neonatal dairy calves despite efforts to decrease the incidence of diarrhea through colostral management, sanitation, and housing. Diarrhea caused by salmonellosis can lead to substantially higher mortality in outbreaks, particularly in calves within the first few weeks of life.[1] Preventing these early-life *Salmonella* infections is particularly difficult because no vaccines are labeled for use in young calves. Extra-label use of commercially available vaccines has not been effective[2] and is anecdotally associated with clinically relevant adverse effects. Colostral immunity stimulated by vaccinating dry cows for *Escherichia coli*, rotavirus, and coronavirus has been used extensively in the dairy and beef industries to protect calves against diarrhea challenge.[3,4] Previous data also indicate that vaccination of dry cows can decrease fecal shedding of *Salmonella* in young calves.[5-7] However, the concept of using dry cow vaccination to decrease clinical disease caused by *Salmonella* in calves has not been well examined.

Administration of a *Salmonella* Newport siderophore-receptor and porin (SRP) subunit vaccine per label directions to cows during the dry period can substantially increase the *Salmonella*-specific colostral titers. When fed to calves within the first hours of life, this colostrum led to a significant increase in the calves' *Salmonella* Newport antibody titers.[8] This finding suggests that dry cow vaccination and subsequent colostrum administration may be a viable method to provide immunity against *Salmonella* in neonatal calves, potentially decreasing the incidence, severity, and mortality of the infection. Therefore, our objective was to evaluate the extent of protection from a *Salmonella* Typhimurium challenge in calves fed colostrum from cows vaccinated with a *Salmonella* Newport SRP subunit vaccine. We hypothesized that administration of colostrum from cows vaccinated with a *Salmonella* Newport SRP subunit vaccine would decrease mortality in calves fed with this colostrum and challenged with *Salmonella* Typhimurium.

2 | MATERIALS AND METHODS

2.1 | Dry cow vaccination

This study was approved by the NC State University Institutional Animal Care and Use Committee (protocol #16-050A). Thirty dry cows (15 control and 15 vaccinated) were given the SRP *Salmonella* Newport bacterin (Zoetis; Parsippany, New Jersey) or saline SC in the neck at cessation of lactation (dry off) and again 3-4 weeks later with 1.5 in, 18 gauge needles. All cows were identified by a colored neckband and housed together on a grass lot with access to a total mixed ration. The cows were vaccinated between March and June 2016 at the NC State Dairy Education Unit, a 200-cow dairy. All mature cows (no heifers were included) that were entering their dry period during this time were eligible to be included, but we ensured that no cows from our previous studies of *Salmonella* vaccination[8,9] were included in either group. Cows were enrolled 2-3 at a time into each group. Two cows were eliminated and replaced because they calved before administration of the booster.

At calving, colostrum from each enrolled cow was collected into gallon bags (Perfect Udder, Dairy Tech, Inc., Greeley, Colorado) within 4 hours of calving, labeled, and frozen at −20°C. A separate aliquot of colostrum from each cow was frozen at −20°C for determination of the colostral anti-*Salmonella* titer. Once colostrum from all 30 cows was collected, the bags were gently thawed, mixed into either the control (CTL) or vaccinated (VAX) pool, and realiquoted in gallon bags and refrozen.

2.2 | Participants

All Holstein bull calves from single (not twin), normal, observed births on the NC State Dairy Education Unit were eligible for inclusion in the study. Any calf that met these criteria was enrolled into the study until 10 calves were enrolled into each group. Enrollment occurred between January and September of 2017. Calves were not randomly allocated to treatment groups before colostrum feeding.

2.3 | Interventions

Calves were fed 4 L of either CTL or VAX colostrum within 4 hours of birth. Randomization of treatments was achieved by mixing the bags of colostrum from the 2 groups of cows in the freezer, and feeders would choose the next available bag when a calf was born.

2.4 | Blinding

Pooled CTL or VAX colostrum was fed to calves and recorded by farm staff so that investigators who performed the inoculation and all assessments were blinded to the treatment. Records of which calf was in each group were maintained on the farm of origin, away from the laboratory animal facilities where the inoculation and assessments took place.

2.5 | Challenge model

Calves were transported to the BSL-2 laboratory animal facilities at 2-3 days of age, and fed 3 L of a commercial milk replacer twice a day by bucket. Each calf was housed in an individual stall in a common room separated by approximately 1-2 m to prevent contact among calves. All calves were infected with 10^9-10^{11} colony-forming units (cfu, range: 6.7×10^9 to 3.4×10^{11}) of *Salmonella* Typhimurium between 10 and 14 days of age. The source was a clinical isolate originally obtained from a calf submitted to the Wisconsin Veterinary Diagnostic Laboratory. A single colony of the *Salmonella* Typhimurium was grown overnight in tryptic soy broth and incubated at 37°C with gentle shaking. The next morning, a volume with the expected concentration of *Salmonella* Typhimurium was mixed with 1 L of milk replacer and 2.5 g of sodium bicarbonate, 2.5 g of magnesium trisilicate, and 2.5 g magnesium hydroxide to alkalinize the abomasum.[10] Slight differences in inoculum dose occurred based on variability in the overnight growth of the isolate. The inoculum in milk was fed to the calf at the morning feeding on day 0. The calf then received the remainder of its feeding (2 L). The milk replacer was mixed with deionized non-chlorinated water to prevent interference with infection because of chlorination. A 1 mL aliquot of the overnight culture was

diluted in sterile phosphate buffered saline 10-fold and plated in triplicate onto 5% Columbia blood agar plates for confirmation of the starting bacterial concentration. The isolate, inoculum dose, and alkalization of the abomasum were chosen based on consistency of disease induction in a pilot study of normal colostrum-fed calves of a similar age.

2.6 | Outcome assessment

The primary outcome assessed was mortality in calves after infection. Any calf that did not eat for 24 hours or was moribund was euthanized for humane reasons. Any calf that died or was euthanized before 4 days after infection was counted toward the mortality in that group. Secondary outcomes also were evaluated to determine if consumption of colostrum from vaccinated cows would impact the titers, clinical disease, or shedding of *Salmonella* in calves after experimental infection. These secondary assessments are described below.

2.7 | *Salmonella* serology

Blood samples were collected from each calf between 24 and 36 hours of age and centrifuged at 1000*g* for 10 minutes to collect serum to determine *Salmonella* titers. Serum and colostrum antibody titers for *Salmonella* Newport were determined as previously described by an ELISA.[8] Personnel performing the ELISA assays were not aware of group assignment for any of the animals. *Salmonella* Newport SRP antigen was coated on ELISA plates (Immulon-2 ELISA plates, Dynatech Laboratories, Chantilly, Virginia) at 250 ng/well in carbonate coating buffer (pH 9.6), and samples were tested in duplicate. After incubation and washing, conjugate sheep anti-bovine immunoglobulin G3 (IgG3) was added to each well. Plates were covered and incubated at 37°C for 1 hour. Plates were washed and developed using 100 μL of 2,2′-azino-di-3-ethyl-benzthiazoline-6-sulfonate (Kirkegaard & Perry, Gaithersburg, Maryland), and absorbance was read at 405/490 nm with an ELISA reader (BioTek ELx405, Winooski, Vermont). Results were reported as sample-to-positive (S:P) ratios. The average of the negative controls' optical density (OD) was calculated and subtracted from all values as a reagent blank. Individual plate variability was addressed by analyzing the OD reading of a known positive and negative OD reading from the same plate. The sample duplicates then were averaged and divided by the positive control average, yielding the S:P titer.

2.8 | Clinical assessments of calves

Calves were assessed twice daily by blinded observers at the time of feeding to determine depression score, rectal temperature, fecal score, milk intake, and eyeball recession. All assessments were performed by a single investigator (Derek Foster) or research technician specifically trained to ensure consistency. The depression score was based on a 0-4 system as follows: 0—normal, no signs of depression; 1—noticeable depression without apparent signs of weakness, slower than normal calf but still raises head when approached and does not appear weak, actively follows observer's movements with a raised head; 2—marked depression with moderate signs of weakness without a substantially altered gait, stands with head lowered, will respond when approached but will return to depressed stance, moves slowly, may display signs of weakness such as incoordination; 3—severe depression with signs of weakness such as a substantially altered gait, obvious weakness, difficulty in moving, raises head only when approached closely; and 4—moribund, unable to rise.[11] Fecal scores were based on a 0-4 scale as follows: 0—normal; 1—semi-formed, pasty; 2—loose, but remains on top of bedding; 3—watery, seeps through bedding; and 4—watery with blood.

2.9 | Clinical sample collection

Blood samples were collected aseptically for a CBC and blood culture each morning, and feces were collected each morning for culture. Any calf still alive on day 4 was euthanized for sample collection. Samples collected at necropsy for culture included ileal contents, ileal tissue, mesenteric lymph nodes, and bile. Ileal tissue also was collected for histology, immunohistochemistry (IHC), and assessment of barrier function in Ussing chambers.

2.10 | Complete blood count

An appropriately filled 2 mL EDTA collection tube was used for the CBC. All samples were checked for clots before being run through the Advia 120 (Siemens Healthineers, Erlangen, Germany). Packed cell volume, plasma protein (PP) concentration, fibrinogen concentration, 100 cell white blood cell differential, and slide review were performed by laboratory technical staff. To determine the fibrinogen concentration, 4 microhematocrit tubes were filled and centrifuged (Heraeus pico 17; Thermo Fisher Scientific, Waltham, Massachusetts) for 1 minute. Two microhematocrit tubes were placed into water bath (58°C) for 3 minutes, and all tubes then were centrifuged for 3 additional minutes. The PP concentration from all tubes was measured using a digital refractometer (Reichert Technologies, Depew, New York), and the fibrinogen concentration was calculated by subtracting the heated PP concentration from the unheated PP concentration. The final result was multiplied by 1000.

2.11 | Salmonella detection

Feces or gut contents obtained from calves were evaluated for the presence of *Salmonella* Typhimurium. Briefly, a swab of fecal or gut contents was used to inoculate XLT-4 (Remel; Thermo Fisher Scientific) and Hektoen Enteric (HE; Remel) plates. Additionally, 10 g of feces was enriched in 90 mL of tetrathionate broth with 200 μL of iodine. The plates were incubated overnight at 37°C, and the tetrathionate was incubated overnight at 42°C. The next day, plates were observed for the presence of morphologically representative colonies (eg, black), and if observed, isolates were streaked for isolation and confirmed to be *Salmonella* using MALDI-TOF mass spectrometry (Vitek MS; Biomerieux, Marcy-l'Etoile, France). If representative colonies were not observed on pre-enrichment plates, the tetrathionate enrichment was inoculated onto XLT-4 and HE plates, which were incubated overnight at 37°C

and evaluated after 24 hours. Again, suspect colonies were streaked for isolation and confirmed.

Blood culture bottles were submitted to the NC State Veterinary Hospital Microbiology & Molecular Diagnostic Laboratory for processing according to standard operating procedures. Five to 10 mL of blood, collected directly into BD Bactec Plus Aerobic Media bottles (BD, Franklin Lakes, New Jersey) were incubated in the Bactec for up to 7 days to assess growth. Bacterial growth was assessed by automated detection of production of carbon dioxide associated with bacterial replication. If positive, the blood culture bottle was removed and evaluated by Gram stain to confirm the presence of Gram-negative rods. Positive bottles also were subcultured onto 5% Columbia sheep blood agar (Remel), and isolates were confirmed to be *Salmonella*.

2.12 | Histologic assessment

Serial sections of formalin-fixed paraffin-embedded ileum were stained using hematoxylin and eosin. The sections were examined by light microscopy and scored in 3 areas. The histologic scoring was as follows: villus blunting—0 = normal villus height, 1 = mild-moderate blunting, 2 = severe blunting; villus epithelial loss—0 = no denuded areas, 1 = <25% of villus denuded, 2 = 25%-50% of villus denuded, 3 = 50%-75% of villus denuded, 4 = >75% of villus denuded; subepithelial villus architecture—0 = normal, 1 = some loss of architecture, 2 = substantial loss of architecture. The sum of these scores was used to compare the CTL and VAX groups. One investigator (Derek Foster) evaluated all slides, and was blinded to animal identity during slide evaluation.

2.13 | Immunohistochemical assessment

Unstained, formalin-fixed paraffin-embedded ileal sections were stained using a mouse monoclonal anti-*Salmonella* antibody and standard IHC techniques. A serial section was included on each slide in which the primary antibody was omitted and replaced with the blocking serum to assess nonspecific staining. Sections were deparaffinized and rehydrated, then treated with a 3% H_2O_2/70% MeOH block followed by a protein block (#MP-7402: normal horse serum, Vector, Burlingame, California). A primary antibody (#5D12A: mouse anti-*Salmonella*, BioRad ABD, Hercules, California) at a 1:2000 dilution was applied for 1 hour. Slides were incubated in the secondary antibody (#MP-7402: anti-mouse IgG, Vector) for 30 minutes. Sections were stained (#SK-4105: ImmPACT DAB peroxidase substrate, Vector) and counterstained (Modified Mayer's Hematoxylin, Richard Allan Scientific, Kalamazoo, Michigan), and slides then were dehydrated and coverslipped. After IHC staining, sections were scored on a 0-4 scale as follows: 0 = no *Salmonella* attached to epithelium or in submucosa, 1 = few *Salmonella* on epithelium, none in submucosa, 2 = 0-25 *Salmonella* in the submucosa per 400× field, 3 = 25-50 *Salmonella* in the submucosa per 400× field, 4 = >50 *Salmonella* organisms in the submucosa per 400× field. One investigator (Derek Foster) evaluated each slide, and was blinded to animal identity during slide evaluation.

2.14 | Assessment of barrier function

For measurement of transepithelial electrical resistance (TER), ileal tissue was harvested at the time of euthanasia, opened on the antimesenteric border, and placed in oxygenated Ringer's solution. The serosal and muscularis layers were stripped from the mucosa, and sheets of ileal mucosa were mounted in a 1.27 cm^2 aperture chamber. The tissue was bathed with circulating, warmed, oxygenated Ringer's solution containing glucose (10 mmol/L serosal) and mannitol (10 mmol/L mucosal) in an Ussing chamber (Physiologic Instruments; San Diego, California). Tissue remained mounted for 60 minutes. Solutions were oxygenated and circulated by gas lift (95% O_2/5% CO_2) and maintained at 37°C by water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges, and the PD was short-circuited using a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between −1.0 and 1.0 mV, tissues were current clamped at ±100 μA for 5 seconds, and the PD was recorded. Transepithelial electrical resistance ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current (Isc). Automated measurements of PD and Isc were taken every 20 seconds over an hour. The mean TER for each tissue starting at 15 minutes was calculated for each calf to account for changes associated with equilibration in the chambers. Glucose was added to the mucosal surface at the conclusion of the hour to induce a current and confirm tissue viability at the conclusion of the hour.

2.15 | Statistical evaluation

For all normally distributed continuous data, a 2-tailed *t* test was used to compare the mean values of the CTL and VAX groups. These outcomes include colostral titers, serum titers, rectal temperature, days fecal culture was positive for *Salmonella*, total leukocyte count, segmented neutrophil count, band neutrophil count, fibrinogen, and TER. For data that were not normally distributed or not continuous (ie, clinical scoring), a Mann-Whitney rank sum test was used to compare the median values of the CTL and VAX groups. These outcomes include depression scores, number of depression scores ≥3, fecal scores, number of fecal scores of ≥3, number of rectal temperature measurements >39.4°C, eyeball recession, milk intake per feeding, days until fecal culture was positive for *Salmonella*, number of positive blood cultures, days until first positive blood culture, days with band neutrophilia, days with abnormally high or low segmented neutrophil count, histology score, and IHC score. The Shapiro-Wilk test was used to determine normality. Pearson correlation was used to evaluate associations among birth weight, time to colostrum feeding in minutes, and inoculum dose with survival. A Fisher's exact test was used to compare the proportion of calves surviving in each group until 4 days of age, the proportion in each group with at least 1 positive blood culture and the proportions of necropsy samples (ileal contents, ileal tissue, mesenteric lymph nodes, and bile) that were culture positive between the 2 groups. A Kaplan-Meier survival analysis was used to compare calf survival between the 2 groups. If a calf died or was euthanized early, data from that calf were included in all analyses unless otherwise noted below. A $P \leq .05$ was considered significant for all comparisons. For all statistically significant

results, the mean difference was calculated to indicate the effect size. Statistical analysis was performed using commercially available software (SigmaPlot 12.5; Systat Software, San Jose, California).

3 | RESULTS

3.1 | Baseline data

The study flow is outlined in Figure 1. No adverse events were associated with vaccination. Neither mean birth weight (P = .05; mean [SD], CTL = 37.7 [3.6] kg, VAX = 42.3 [5.8] kg) or mean time from birth to colostral feeding (P = .22; mean [SD], CTL = 153 [43] minutes, VAX 131 [37] minutes) were significantly different. Similarly, the mean age of inoculation was not different between the 2 groups (P = .30; mean [SD], CTL = 12.3 [4.3] days, VAX = 10.5 [3.1] days). Inoculum dose did not vary between groups (P = .91; median [interquartile range, IQR], CTL = 1.6 [1.0, 8.6] $\times 10^{10}$ cfu, VAX = 1.4 [1.0, 8.6] $\times 10^{10}$ cfu; Table 1).

TABLE 1 Birth weight, colostrum feeding (mean with SD), and inoculation variables (median with interquartile range) of calves that received control (CTL) and vaccinated (VAX) colostrum

	CTL	VAX	*P* value
Birth weight (kg)	37.7 (3.6)	42.3 (5.8)	.05
Age of colostrum feeding (min)	153 (43)	131 (37)	.22
Age at inoculation (d)	12.3 (4.3)	10.5 (3.1)	.30
Inoculum dose ($\times 10^{10}$ cfu)	1.6 (1.0, 8.6)	1.4 (1.0, 8.6)	.91

3.2 | Calf survival

In the CTL group, 4/10 calves died or were euthanized before day 4, and 5/10 calves in the VAX group died or were euthanized early (Figure 2 and Table 2). Neither the proportion of calves surviving nor the median survival time was significantly different between the 2 groups (Table 2). Survival was not associated with time to colostral feeding, birth weight, or inoculum dose.

3.3 | Serology

The VAX colostrum titers were significantly higher than the CTL titers (P = <.001). The CTL mean (SE of the mean, SEM) was 0.816 (0.04) and the mean (SEM) of VAX titers was 1.344 (P = .05; Figure 3 and Table 2). Calf titers also were significantly different (P = <.001). The CTL mean (SEM) titer was 0.423 (0.02) and the VAX mean (SEM) titer was 0.852 (P = .02; Figure 3 and Table 2).

3.4 | Clinical assessments

The median depression score for both groups was 1 with a IQR of 0-2 (P = .18; Table 2). The median (IQR) number of days that calves had a

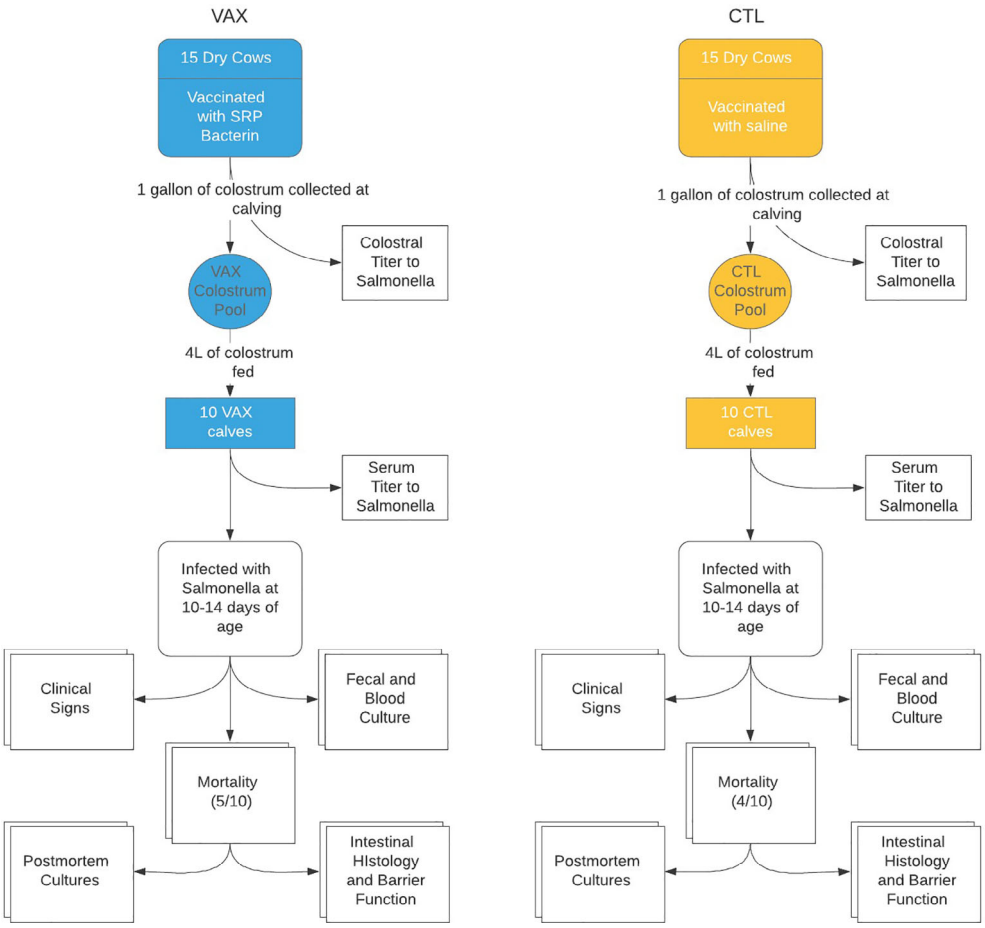

FIGURE 1 Study flow of vaccinated cows and calves in the study

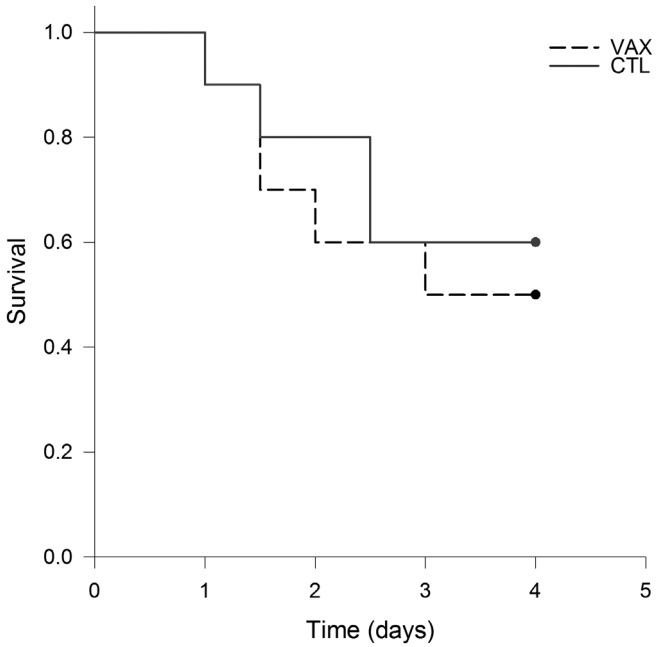

FIGURE 2 Survival curve of calves fed colostrum from either cows vaccinated with a *Salmonella* Typhimurium subunit bacterin (VAX) or saline control (CTL). Time 0 indicates day of inoculation, and all surviving calves were euthanized on day 4. There was no significant difference between the 2 groups

depression score of ≥3 was 1.5 (0, 3) days in the CTL group and 1 (0, 2) day in the VAX group (P = .61; Table 2). The median (IQR) fecal score was 2 (1, 3) for both groups (P = .07), and the median (IQR) number of fecal scores ≥3 was 2.5 (0.75, 6—CTL; 0.75, 5.25—VAX) days for both groups (P = .97; Table 2).

The daily mean (SEM) rectal temperature was not different between the 2 groups (CTL = 39.7 [0.12]°C, VAX = 39.5 [0.12]°C; P = .23; Table 2). The mean (SEM) number of rectal temperature measurements consistent with a fever (>39.4°C) also was not different between groups (CTL = 4.5 [0.8], VAX = 3.3 [0.9]; P = .33; Table 2). Median (IQR) eyeball recession was not different between the 2 groups (CTL = 0 [0, 1.4], VAX = 0 [0, 0.5]; P = .56; Table 2). Median (IQR) milk intake per feeding was not different between groups (CTL = 2 [1, 3] quarts, VAX = 3 [0.9, 3] quarts; P = .45; Table 2).

3.5 | Fecal, blood, and tissue culture

All calves in both groups had at least 1 positive fecal culture during the study. The mean (SEM) number of days that calves were fecal positive for *Salmonella* was not different (CTL = 3.3 [1.6], VAX = 2.7 [0.95]; P = .31; Table 2). Similarly, the median (IQR) number of days until the first positive fecal culture was not different between the 2 groups (CTL = 1 [1] days, VAX = 1 [1] days; P = .37; Table 2). For blood cultures, 7/10 CTL and 4/9 VAX (1 calf was not included because it died within the first 24 hours) calves were positive for *Salmonella* at some point after infection (P = .65; Table 2). There was no difference in the median (IQR) number of blood culture positive days (CTL = 1 [0, 2] days, VAX = 1 [0, 1.25] days; P = .60) or days until positive (CTL = 1 [1, 3] days, VAX = 1 [1, 2.5] days, P = .95; Table 2).

At necropsy, ileal contents were positive for *Salmonella* in 9/9 CTL and 7/9 VAX calves (P = .47, no ileal contents could be collected from 1 calf in each group), and ileal tissue was culture positive for 10/10 CTL and 8/10 VAX calves (P = .47; Table 2). Mesenteric lymph nodes were culture positive in 10/10 CTL and 9/10 VAX calves (P = 1.0; Table 2). Bile was culture positive in 6/10 CTL and 6/9 VAX calves (P = 1.0, no bile could be obtained from 1 VAX calf; Table 2).

3.6 | Hematology

Mean total leukocyte counts were not different on any day of the study between the 2 groups. Similarly, no significant differences were found between the 2 groups in mean fibrinogen concentration, segmented neutrophil count, or band neutrophil count on any day of the study. No significant differences were found in the median (IQR) number days with band neutrophilia (CTL = 3.5 [1.75, 4] days, VAX = 3 [1, 4] days; P = .50) or mean (SD) abnormal segmented neutrophil count (CTL = 2.3 [0.3] days, VAX = 2.3 [0.4] days; P = 1.0; Table 2).

3.7 | Transepithelial electrical resistance

The mean (SEM) TER of CTL calves was 67.1 (13.8) $\Omega \cdot cm^2$ and 78.8 (10.6) $\Omega \cdot cm^2$ (P = .51; Table 2) in VAX calves, suggesting that there was no difference in epithelial barrier function between the 2 groups.

3.8 | Histology and IHC

The median (IQR) combined histology score was not different between groups (CTL = 3.0 [0, 6], VAX = 3.5 [1, 7]; P = .52), nor was the IHC score (CTL = 2 [1.75, 3.25], VAX = 2 [1, 2]; P = .24; Table 2). Examples of moderate to severe histologic and IHC scores are presented in Figures 4 and 5.

4 | DISCUSSION

We found no difference in mortality between calves inoculated with *Salmonella* Typhimurium that received colostrum from cows that were given *Salmonella* Newport bacterial extract during the dry period and those that received colostrum from sham-vaccinated cows. Furthermore, no statistically significant or clinically relevant differences in clinical signs or necropsy were found between the 2 groups of calves. Yet, as shown in previous studies,[8,9] administration of *Salmonella* vaccines during the dry period can induce significant increases in both colostral titers and serum titers in calves fed this colostrum from vaccinated cows. Despite the increased titers in VAX calves, we found no evidence of protection after inoculation with *Salmonella* Typhimurium.

Our study assessed clinical findings including mortality, depression, fecal consistency, and dehydration. Mortality approached 50% in both groups and was associated with significant depression in most calves. All calves developed diarrhea, often severe, but few calves

TABLE 2 All comparisons between the control (CTL) and vaccinated (VAX) groups and the difference between means of the statistically significant findings. Results of parametric tests are reported as mean (SE of mean), and results of nonparametric tests are presented as median (interquartile range)

	CTL	VAX	*P* value	Significance test	Difference in means
Colostral titer	0.813 (0.04)	1.344 (0.05)	<.001	*t* test	0.531
Serum titer	0.423 (0.02)	0.852 (0.02)	<.001	*t* test	0.429
Calf mortality	4/10	5/10	1	Fisher's exact test	
Survival time	4 (2.25)	3.5 (1.5, 4)	.68	Mann-Whitney rank sum test	
Depression score	1 (0, 2)	1 (0, 2)	.18	Mann-Whitney rank sum test	
Days with depression score >3	1.5 (0, 3)	1 (0, 2)	.61	Mann-Whitney rank sum test	
Fecal score	2 (1, 3)	2 (1, 3)	.07	Mann-Whitney rank sum test	
Fecal scores >3	2.5 (0.75, 6)	2.5 (0.75, 5.25)	.97	Mann-Whitney rank sum test	
Rectal temperature	39.7 (0.12)	39.5 (0.12)	.23	*t* test	
Rectal temperature measurements >39.4°C	4.5 (0.8)	3.3 (0.9)	.33	*t* test	
Eyeball recession	0 (0, 1.4)	0 (0, 0.5)	.56	Mann-Whitney rank sum test	
Milk intake per feeding	2 (1, 3)	3 (0.9, 3)	.45	Mann-Whitney rank sum test	
Days fecal positive	3.3 (0.5)	2.7 (0.3)	.31	*t* test	
Days until first positive fecal culture	1 (1, 1)	1 (1, 1)	.37	Mann-Whitney rank sum test	
Percentage of calves positive on blood culture	7/10	4/9	.65	Fisher's exact test	
Number of positive blood cultures	1 (0, 2)	1 (0, 1.25)	.6	Mann-Whitney rank sum test	
Days until first positive blood culture	1 (1, 3)	1 (1, 2.5)	.95	Mann-Whitney rank sum test	
Proportion of ileal contents positive	9/9	7/9	.47	Fisher's exact test	
Proportion of ileal tissue positive	10/10	8/10	.47	Fisher's exact test	
Proportion of mesenteric lymph nodes positive	10/10	9/10	1	Fisher's exact test	
Proportion of bile cultures positive	6/10	6/9	1	Fisher's exact test	
Number of days with a band neutrophilia	3.5 (1.75, 4)	3 (1, 4)	.5	Mann-Whitney rank sum test	
Number of days with an abnormal neutrophil count	2.3 (0.4)	2.3 (0.4)	1	*t* test	
Transepithelial electrical resistance	67.1 (13.8)	78.8 (10.6)	.51	*t* test	
Histology score	3 (0, 6)	3.5 (1, 7)	.52	Mann-Whitney rank sum test	
IHC score	2 (1.75, 3.25)	2 (1, 2)	.24	Mann-Whitney rank sum test	

Abbreviation: IHC, immunohistochemistry.

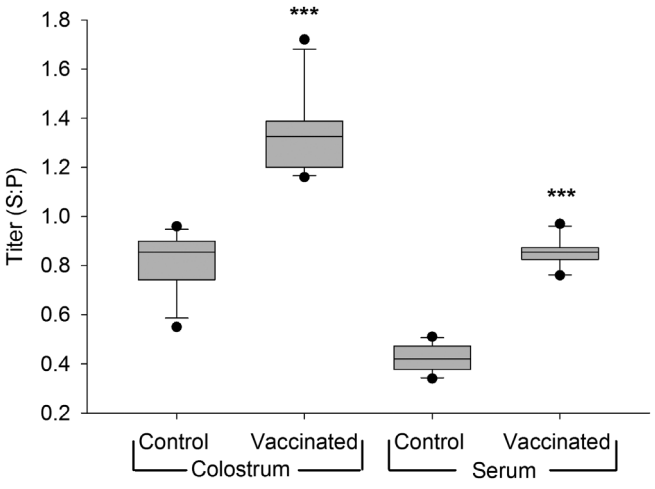

FIGURE 3 Colostral titers from cows vaccinated twice during the prepartum period with a *Salmonella* Typhimurium subunit bacterin or saline control, and serum titers from calves fed pooled colostrum from these cows. S:P, sample-to-positive ratio. ***$P < .001$

were clinically dehydrated based on eyeball recession. This finding may be a consequence of the relatively short course of disease, and because most calves continued to drink most of their daily milk feedings. From these findings, the vaccine does not appear to lessen the severity or duration clinical disease in calves exposed to *Salmonella*.

Septicemia is a common complication to enteric salmonellosis in calves.[12,13] Both CBC and blood cultures were evaluated to assess the systemic inflammatory response and sepsis. Although only 44% VAX calves were blood culture positive compared to 70% in the CTL group, this difference was not significant. These results are similar to those of a previous study of experimental *Salmonella* Typhimurium infection in which 9/15 calves were blood culture positive.[13] Furthermore, the time to first positive blood culture, total number of positive blood cultures, and CBC findings suggest that the vaccine did not effectively prevent septicemia associated with *Salmonella* infection in the calves.

Invasion of the epithelial barrier is a well-recognized consequence of salmonellosis that is a mediator of intestinal inflammation, diarrhea,

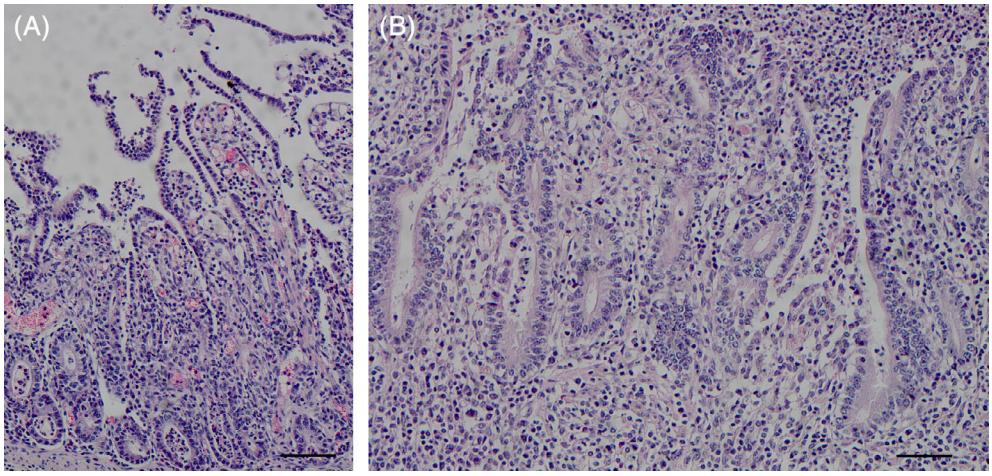

FIGURE 4 Histologic section of the ileum from calves with A, moderate and B, severe damage including villus blunting, loss of epithelium, and neutrophil migration. Hematoxylin and eosin staining. ×400 magnification. Bar = 50 μm

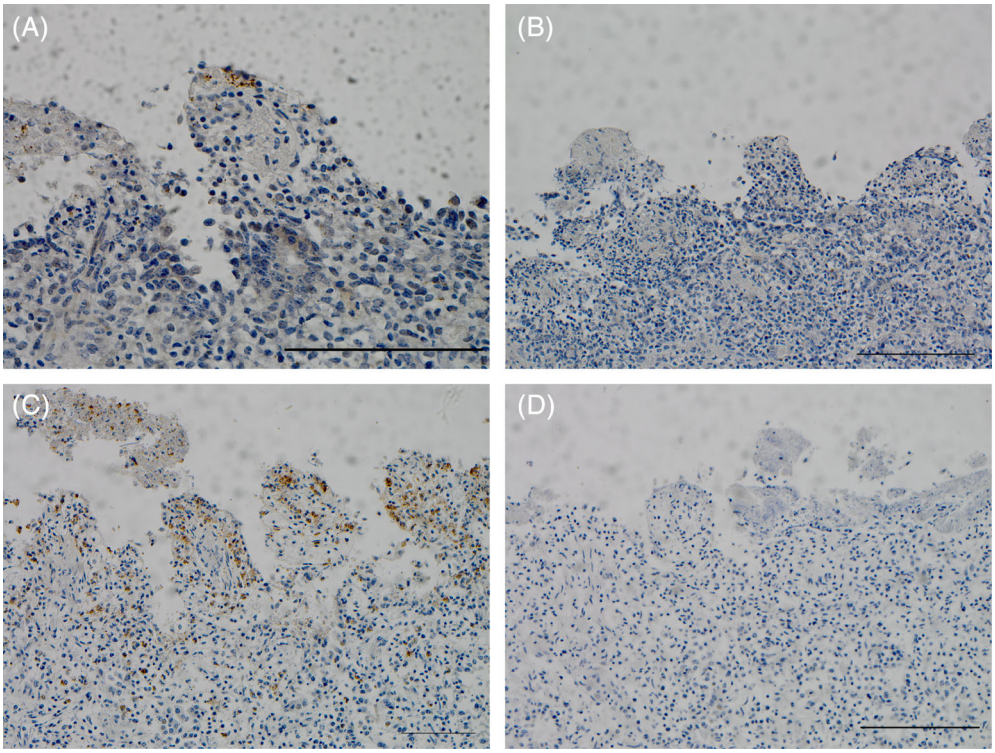

FIGURE 5 Immunohistochemical staining of ileal sections with A, moderate and C, severe invasion of *Salmonella*. Isotype controls are included for both sections (B and D). ×1000 magnification. Bar = 50 μm

and development of long-term carriers.[14,15] To evaluate invasion of *Salmonella* into deeper tissues and potential development of carrier status, culture of intestinal tissue, mesenteric lymph nodes, and bile was performed at necropsy. The majority of these samples were positive in both groups, indicating rapid spread from the intestinal lumen into the submucosa, draining lymph nodes, and gall bladder. These findings are supported by the histologic, immunohistochemical and ex vivo assessments of intestinal damage. Spread of *Salmonella* in calves is primarily via the fecal oral route.[12] Therefore, reduction in fecal shedding by calves could be of benefit in controlling the infection on farms. Unfortunately, there was no measureable impact of feeding colostrum from vaccinated cows on the number of calves shedding *Salmonella* or the number of days that calves were fecal positive for *Salmonella*. Similar numbers of calves still were culture positive at necropsy as well.

Ussing chambers have been used for over 50 years to maintain the viability of epithelia ex vivo in order to investigate ion transport and barrier function, leading to fundamental understanding of the mechanisms of diarrhea.[16] With the Ussing chamber, resistance to movement of ions across the epithelium can be calculated as the reciprocal of the measured conductance. This measurement (TER) provides a sensitive measure of tissue integrity,[16] because changes are almost exclusively a result of loss of intercellular tight junctions and increases in paracellular movement of ions.[17] Transepithelial electrical resistance has been used to assess epithelial cell loss and damage from a variety of causes including ischemia, bile injury, and infection.[18-20] Our finding that there was no difference in TER between the CTL and VAX calves suggests similar severity of epithelial damage, which is supported by the histologic scoring.

Although slightly lower, the colostral and calf titers found in our study were similar to what was described previously.[8] These findings along with another study demonstrating colostral transfer of immunity using a different commercially available vaccine[9] suggest that dry cow vaccination is an effective means of providing passive transfer of antibodies against *Salmonella* to neonatal calves. In our study, this finding should be interpreted with caution, because it was not the primary outcome of interest, and could be because of chance given the number of ancillary tests.

Some prior evidence suggests that colostral immunity might have some benefit in protecting calves against *Salmonella.* In a previous study, vaccination of cows with a killed bacterin at 2 months and again 1 month before calving decreased shedding of *Salmonella* after PO challenge with *Salmonella* Typhimurium at 2 days of age as compared to calves born to unvaccinated cows.[6] However, the study did not evaluate the effects of vaccination on clinical signs or calf mortality. In another study done at a large California dairy that had a high prevalence of salmonellosis, 150 cows received 2 doses of a killed (autogenous) *Salmonella* Montevideo bacterin before calving, another 150 cows received a single dose of a modified live *Salmonella* Choleraesuis vaccine and 150 cows were unvaccinated controls. Calves that received colostrum from cows that had been given the modified live vaccine had a significant reduction in *Salmonella* shedding as compared to calves from the other 2 groups, but there was no significant difference in mortality.[7] In another study, a group of cows received a killed *Salmonella* Typhimurium vaccine at 7 weeks and again at 2 weeks before calving whereas another group was left unvaccinated.[5] Calves were left with their dam for 48 hours to ingest colostrum, and then were given a PO *Salmonella* Typhimurium challenge at 5 days of age. Mortality in the calves that received colostrum from vaccinated calves was only 10% (2 of 20) as compared to 58% (7 of 12) in calves that received colostrum from unvaccinated cows. Calves fed vaccinated colostrum also had significantly shorter periods of *Salmonella* shedding in feces after challenge, and after a 28-day period, these calves were less frequently culture positive for *Salmonella* from the gastrointestinal tract (including ruminal, cecal, and colon contents) and mesenteric lymph nodes. The PO administration of *Salmonella*-specific egg yolk antibodies (IgY) also has been shown to confer dose-dependent protection against a *Salmonella* Dublin and a *Salmonella* Typhimurium challenge in calves suggesting that the presence of mucosal antibody can protect against disease.[21]

It is unclear why no differences were found for any measured outcomes despite increased titers in the VAX group. Little data exist about what constitutes a protective titer in calves, but potentially the titers in our study were not high enough to be protective. Additionally, we did not measure titers at the time of infection, and the reported titers may not truly reflect the status of the calves at 2 weeks of age. In most of the studies discussed above, *Salmonella* challenge occurred between 2 and 5 days of age, and perhaps protection from colostral immunity had waned significantly by the time we challenged the calves in our study (days 10-12). The rate of decay for colostral antibodies does appear to vary for different pathogens,[22] and the persistence of colostrum-derived *Salmonella* immunoglobulins from this subunit vaccine is unknown. If rapid immunoglobulin elimination is the reason for the lack of protection, using passive transfer of immunity to prevent salmonellosis in neonatal calves is unlikely to be a successful strategy.

We only assessed humoral immunity. However, cell-mediated immunity also is critical to protection from *Salmonella*.[12] Although this subunit vaccine can stimulate cell-mediated immunity in mice,[23] it is unknown if it is transferred to calves via colostrum. We chose to use pooled colostrum so that each calf would receive the same concentration of *Salmonella* antibodies and limit potential variability. One disadvantage of this design is that it necessitated the use of frozen colostrum, which would negate any potential benefit from maternally derived T cells. Furthermore, the absorption and activity of colostral leukocytes may be decreased when colostrum is from a cow other than the calf's dam, but this situation has not yet been fully studied. Some evidence in swine indicates cell-mediated immunity was decreased in piglets immediately cross-fostered to other sows after birth.[24] Colostral leukocytes can be absorbed through the intestine of the calf primarily via the follicle-associated epithelium of Peyer's patches.[25] The majority of these cells are T-lymphocytes (both CD4+ and CD8+) that once through the intestinal barrier can migrate along the lymphatics and recirculate to different organs including the liver and spleen.[25,26] Although the exact function of these maternally derived lymphocytes is not clear, increasing evidence suggests that they are involved in the early immune function of the calf.[27,28] These cells are likely important in the development of neonatal monocytes, allowing more effective presentation of antigen as well as development and activation of neonatal lymphocytes.[29,30] A recent study also suggested that maternal colostral cells had long-term effects on the development of the calf's immune system.[31] Calves that received whole colostrum had stronger responses to vaccination 6 to 10 months post-colostrum feeding as compared to calves fed cell-free colostrum. This observation suggests that these maternal cells may play a role in "training" or "priming" the developing immune system of the calf. Therefore, it is possible that the feeding of fresh colostrum to calves from cows vaccinated for *Salmonella* Newport would have produced better protection in this challenge model.

Finally, the failure of protection could be a consequence of the overwhelming infection. The isolate used was obtained from a necropsy sample, and was associated with substantial mortality in our study. Potentially, some protection may have been seen had a less pathogenic isolate been used. Furthermore, the dose of *Salmonella* administered may be higher than that typically encountered in the clinical situation. Unfortunately, other isolates and lower inoculation doses without abomasal alkalization did not reliably induce clinical disease in a small pilot study before this trial. The 2 cycles of freezing and thawing of the colostrum could have decreased the IgG content of the colostrum that was fed, and decreased any protective effect in the calves. Regardless, the colostrum from vaccinated cows did induce a significant increase in serum titers in the VAX group. We did not assess transfer of passive immunity in the calves. Although every effort was made to optimize transfer of colostral antibodies in both groups, there could have been differences in the total IgG transferred, which may have influenced our results.

Several limitations to this study limit the conclusions that can be drawn. First, the cows were not randomly allocated to VAX or CTL groups. This could have inadvertently biased the overall colostral quality in 1 group because this was not assessed. Second, calves were not randomly allocated to treatment groups before the study. Because treatments were randomly applied to the calves as they were born, this approach should have limited the bias introduced by this error, but we cannot rule out the possibility that this approach inadvertently influenced our findings. Measurement of passive transfer of immunity in the calves would have provided some assurance that the lack of randomization did not bias our findings, but this measurement was not done. Finally, the small sample size limits the conclusions that can be drawn because the study was underpowered to demonstrate differences in mortality. We erroneously calculated sample size by treating mortality as a continuous instead of a binary variable. Because of this error, the likelihood of missing a difference in mortality when a difference truly exists is increased. The lack of appropriate randomization and sample size calculation preclude the application of these findings to clinical practice, but the results still are valuable to inform future investigations.

Administration of the *Salmonella* Newport bacterin to dry cows did not provide protective immunity to calves in our study despite increased titers. Additional well-controlled blinded clinical trials on farms with substantial *Salmonella* burdens will be necessary to determine if these titers are protective in clinical outbreaks of salmonellosis.

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CONFLICT OF INTEREST DECLARATION

Geof Smith and Derek Foster received funding for this study and previous research by Zoetis. Geof Smith serves as Associate Editor for the Journal of Veterinary Internal Medicine. He was not involved in review of this manuscript.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the North Carolina State University IACUC.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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