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Randomized controlled trial comparing the immunogenicity of experimental *Salmonella* Dublin siderophore receptor vaccines in calves

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OBJECTIVE
To describe immune responses following administration of experimental *Salmonella* Dublin siderophore receptor protein (SRP) vaccines in Holstein heifer calves with adequate passive antibody transfer.

METHODS
Calves were randomly assigned to receive placebo, vaccination with *S* Dublin SRP in adjuvant A, or vaccination with *S* Dublin SRP in adjuvant B at 7 ± 3 days of age and 3 weeks later. Before each vaccination, 4 and 8 days after the second vaccination (postvaccination), and 61 to 91 days postvaccination, *S* Dublin antibody titers were measured. Blood mononuclear cells isolated from blood collected 4 and 8 days postvaccination were stimulated with *S* Dublin SRP antigen (1 or 5 µg/mL) or positive or negative controls, then analyzed to quantify *S* Dublin SRP–responsive cells. Cultures of blood mononuclear cells were similarly stimulated to quantify interferon-γ (IFN-γ)-producing and IL-17–producing cells. The trial spanned September 4, 2022, through January 15, 2023.

RESULTS
78 calves were enrolled. Vaccinates had significantly higher IFN-γ–producing cells and IFN-γ and IL-17 concentrations at 4 and 8 days postvaccination, except IFN-γ concentration at day 4 after stimulation with 1 µg/mL. Vaccinates also had higher *S* Dublin titers at 8 and 61 to 90 days postvaccination. No differences in health events were noted.

CONCLUSIONS
Vaccination can induce *S* Dublin SRP–specific humoral and cellular immune responses in Holstein heifer calves.

CLINICAL RELEVANCE
Vaccination with SRP vaccines resulted in immune responses that may help mitigate *S* Dublin infection. Further research is needed to determine whether vaccination will be protective against *S* Dublin challenge.

Keywords: bovine, *Salmonella* Dublin, siderophore receptor protein (SRP) vaccine, cell-mediated immunity, humoral immunity

Salmonella enterica serotype Dublin is a common cause of calf illness, with high morbidity and mortality rates in affected animals, and is endemic in many regions of the US and Canadian dairy industries.[1–3] A 2016 report[4] from the US National Veterinary Services Laboratory found that *S* Dublin was the most isolated *Salmonella* serotype obtained from ill cattle in the US. This serotype is also considered to be host adapted in bovine. Therefore, carrier animals that appear normal can be intermittent shedders and a source for maintaining the infection within a herd.[3] As such, finding mechanisms to protect naïve animals from clinical disease can reduce losses of animals and improve animal welfare in dairy herds. This includes maximizing transfer of passive immunity, maintaining high standards of hygiene, removing calves from the maternity environment as quickly as possible to reduce exposure of *S* Dublin

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bacteria shed by carrier dams, and effective vaccination of dams and calves early in life.[3]

Effective vaccination against *Salmonella* species requires the production of both humoral and cell-mediated immune responses as the organism can survive within macrophages.[5–8] To eliminate intracellular *Salmonella*, macrophages must become activated via interferon-γ (IFN-γ) released from T-helper (Th)-1 lymphocytes. Interleukin-17 is produced by Th17 cells and plays a key role during *Salmonella* infections by triggering inflammation and recruitment of inflammatory cells early in the course of infection as well as promoting the upregulation of antimicrobial molecules and optimizing neutrophil function.[5]

Traditional killed vaccines have failed to stimulate effective cell-mediated immune responses,[9–11] so alternative vaccine technologies are needed. A possible strategy to induce effective immunity is to target the iron-acquisition system of *S* Dublin. Iron is an essential nutrient of all gram-negative bacteria. In low-iron environments, such as mammalian tissues, *Salmonella* manufacture and excrete ferric iron chelators called siderophores. These low-molecular-weight proteins bind to iron and transport it back to the bacterium. Siderophore receptor proteins (SRP) are iron-regulating outer membrane proteins and are responsible for transporting siderophore-iron complexes into the bacterial cell for use. Vaccines that utilize SRP from *S* Dublin should restrict the transportation of iron into the bacterium, thereby starving it of this required nutrient, resulting in cell death. Currently, there are 3 licensed SRP subunit vaccines (Vaxxinova US Inc) available for use in cattle that have been used for immunization against *Escherichia coli* O157:H7,[12] *Klebsiella pnuemoniae*,[13] and *S* Newport.[14,15] The vaccines used in the study reported here are experimental subunit vaccines composed of purified extracts of SRP from *S* Dublin. The objective of this work was to describe the immune responses stimulated by experimental *S* Dublin SRP vaccines in Holstein heifers.

Methods

Overview of experimental method

Prior to the initiation of animal work, the study was approved by Iowa State University's IACUC (protocol #22-157). The trial was initiated on September 4, 2022, and concluded on January 15, 2023.

A commercial dairy farm with no history of disease associated with *S* Dublin and not using any *Salmonella* or SRP vaccines in adult animals or calves was recruited to participate in this study. The infection status of the herd was verified by the herd's veterinarian, who had multiple years of experience with the operation. Historical diagnostic testing completed by the veterinarian had not uncovered any *S* Dublin cases. The health status of the herd and the participants was monitored throughout the study through the evaluation of herd records. All health events were captured by farm staff in the farm's herd management software (Dairy Comp 305; Valley Ag Software). Farm staff was also trained to watch calves on the days of vaccination and to notify trial personnel if they noticed any problems. Following completion of the study, health events for study participants were captured from the software through 90 days of age and compared.

To confirm *S* Dublin–negative status of the lactating herd, bulk tank milk from the herd was collected 4 times throughout the study at approximately 3-week intervals and then 1 and 2 months after the study was completed. The milk samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory and tested via a commercial *Salmonella* ELISA (PrioCHECK; ThermoFisher). Additionally, all trial calves were checked for infection status via the same ELISA kit on blood collected between 90 and 120 days of age. *Salmonella* ELISA testing was completed using standardized laboratory protocols. A value of ≥ 35% positivity was used to indicate a positive result per the recommendations of the test kit manufacturer.

Sample size calculations were performed using freely available software,[16] using cellular immune responses as the main study outcome. To determine differences between vaccinated and placebo groups, we estimated that the frequency of antigen-responsive cells would increase 2-fold based on previous vaccine studies[17–19] conducted in calves of similar ages. Assuming a significance level of $\alpha = 0.05$ and a power of 80%, 60 calves would need to be enrolled (20 calves/treatment). To account for attrition, a minimum of 24 calves/treatment were enrolled. On the final date of calf enrollment, all eligible calves were vaccinated. This, along with study attrition, resulted in different final numbers of calves in each group.

Vaccines for this study were comprised of *S* Dublin SRP and an adjuvant. The SRP were produced as previously described except that *S* Dublin was used instead of *E coli*.[20] Briefly, *S* Dublin was grown in an iron-restricted medium, and the cells were collected by tangential flow filtration. The cells were then disrupted with a homogenizer and the solution solubilized with an anionic surfactant. The SRP were collected by centrifugation, washed, and concentrated by tangential flow filtration against physiological saline. The final antigen was then mixed with either an oil-in-water adjuvant (adjuvant A) or a polymer-based adjuvant (adjuvant B) to compare immune responses between the 2 adjuvants and vaccinates with either adjuvant and controls. The vaccines were shipped from the manufacturer on ice to the laboratory of 1 of the authors (PJG), where it was stored according to label directions throughout the duration of the study. On the days when the vaccine was to be administered, it was placed in a cooler with ice packs for transport and only removed long enough for vaccine administration. Following vaccination, the vaccine was transported back to the laboratory.

Study cohort

Prior to study initiation, the randomization schedule was completed using a spreadsheet program (Excel; Microsoft Corp) by 1 of the authors

(PJG), with calves enrolled assigned in birth order if they had sufficient passive transfer of colostral antibodies. Calves were enrolled once per week over the course of 8 weeks if they had a serum protein value ≥ 5.5 mg/dL using a handheld digital refractometer (model DD-3; MISCO) from blood samples collected between 1 and 7 days of age and were healthy at the time of first vaccination. The calves were enrolled into 1 of 3 treatment groups (Gr): (1) placebo (sterile saline only; Gr1), (2) *S* Dublin SRP in adjuvant A (Gr2), or (3) *S* Dublin SRP in adjuvant B (Gr3). At 7 ± 3 days of age and 3 weeks later, calves from Gr2 and Gr3 were vaccinated SC in the neck with 1 mL of their assigned vaccine, whereas Gr1 received 1 mL of saline.

Prior to each vaccination, at 4 and 8 days after the second vaccination and 61 to 91 days after the second vaccination (approx 90 to 120) days of age, 10 mL of blood was collected from the jugular vein in red-top tubes (Vacutainer; Becton, Dickinson, and Co) for antibody (Ab) titer analysis. Blood tubes were immediately placed on ice and transported back to the laboratory of 1 of the authors (PJG), where serum was harvested. Serum samples were stored at −80 °C until the trial was complete, at which time they were shipped overnight to the laboratory of the sponsor. Additionally, at 4 and 8 days after the second vaccination, 10 mL of whole blood for isolation of peripheral blood mononuclear cells was collected from the jugular vein in cell preparation tubes (CPT tubes; Becton, Dickinson, and Co) for cell-mediated immunity analysis. Following collection, CPT tubes were placed on ice and transported to the laboratory of 1 of the authors (JLM). Cell-mediated immunity assays were run upon arrival at the laboratory. Immediately following each vaccination, trial personnel visually monitored calves for signs of anaphylactic-type reactions for a period of 1 hour, and farm staff monitored them afterward.

Sample analysis

All laboratory personnel conducting immune assays were blinded to the treatment assignment of the individual animals.

The serological response to vaccination was evaluated using an ELISA using the *S* Dublin SRPs as the capture molecule for the assay at the laboratory of the sponsor. Briefly, 96-well polystyrene plates (Immulon-2HB; Immunochemistry Technologies) were coated with *S* Dublin–SRP antigen at 250 ng/well, diluted in a carbonate coating buffer at pH 9.6. The plates were then covered and incubated overnight at 4 °C. Plates were then emptied and patted dry, blocked using 200 µL/well of 1% polyvinyl alcohol/PBS, covered, and incubated 1 hour at 37 °C. Each serum sample was diluted 3-fold in 1% polyvinyl alcohol/PBS from 1:500 to 1:121,500 and tested in duplicate. All plates contained 2 wells of a 1:500 target dilution of known positive control sera, which served as an internal plate control to ensure a valid test. After serum addition, plates were incubated for 1 hour at 37 °C and washed 3 times in Tris-PBS and patted dry. A goat anti-bovine conjugate was diluted 1:40,000, and 100 µL was applied to each well of the plate and incubated for 1 hour at 37 °C. The plates were then washed 3 times in Tris-PBS and patted dry. Plates were developed by adding 100 µL/well of 2,2' azino-di-(3-ethyl-benzthiazoline-6-sulfonate) 2-component peroxidase substrate and incubated for 12 to 15 minutes at room temperature. Plates were then read on an ELISA plate reader at 405/490 nm. The detection threshold was defined as the median value of all blank wells (0.01425). To estimate the dilution at which the ELISA values for each sample would fall below the established threshold, a log-log linear model was fitted. The log-log linear range of the curve was visually determined to range from a value of 0.85 to when the value dropped below double the average of the blanks for the plate. All dilutions of 1:121,500 and greater were excluded. A model with a common slope was fit to predict the log of the corrected ELISA value (the raw value minus the mean of the blanks on the plate) by the log of the dilution factor with the individual effect modifying the intercept. The point at which each fitted line crossed the threshold (0.01425) was then calculated and assigned as the titer value.

To determine cell-mediated immunity responses, bovine IFN-γ assays (ELISpot; MabTech Inc) were used to quantify the number of SRP-responsive immune cells. Blood mononuclear cells were resuspended in cell medium (complete RPMI; ThermoFisher) supplemented with 10% fetal bovine serum, nonessential amino acids, essential amino acids, sodium pyruvate, 2-mercaptoethanol, and penicillin/streptomycin. Cells were then plated at a concentration of 5 X 10^5 cells/well in duplicate ELISpot wells and stimulated with 1 or 5 µg/mL of *S* Dublin SRP antigen. Two separate concentrations were utilized in the assays to explore a range of effective antigen concentrations and ensure sufficient stimulation of the cultured cells. Negative control wells received media only; positive control wells were stimulated with 1 ug/mL concanavalin A (MilliporeSigma). Cells were incubated at 37 °C with 5% CO_2 for 18 hours. Plates were developed using alkaline phosphatase per the manufacturer's instructions and read on an ELISpot reader (MabTech Inc). The results were reported as spot-forming units/10^6 stimulated cells.

In parallel cultures, peripheral blood mononuclear cells were also stimulated with *S* Dublin SRP antigens with supernatants used to quantify concentrations of IFN-γ (Invitrogen IFN gamma Bovine ELISA Kit; ThermoFisher) and IL-17 (Invitrogen IL-17A Bovine ELISA Kit; ThermoFisher). Blood mononuclear cells were plated at 5 X 10^5 cells/well in triplicate wells of a round-bottom, 96-well plate. Negative control wells received media only, stimulated wells received either 1 or 5 µg/mL SRP antigen, and positive control wells were stimulated with 1 µg/mL concanavalin A. Plates were incubated for 48 hours at 37 °C in 5% CO_2. Supernatants were pooled from triplicate wells and frozen at −80 °C until analysis. Concentrations of IL-17 and IFN-γ were measured in duplicate per the manufacturer's instructions.

Statistical analysis

Statistical analysis was completed using freely available statistical software (R, version 4.3.0; R Foundation for Statistical Computing) with an added package for streamlining and plotting (Tidyverse, version 2.0.0). Descriptive statistics were compared using ANOVA for the numeric variables and chi-square or proportion tests for the health event data. Enzyme-linked immunosorbent assay titers and cell-mediated immune responses for placebos and vaccinates were compared at each time point independently to reduce the compounding impact of developmental shifts in immune response. Because titer data are, by their nature, logarithmic, log transformation was used for all titer analyses. This was determined prior to data analysis, and a visual inspection confirmed that the log-transformed data more closely met normality assumptions than untransformed data. As a first step to assess adjuvant effects, an ANOVA was conducted on the log-transformed values followed by the Tukey honestly significant difference test for each comparison (each adjuvant vs control and the adjuvants against each other). Because the 2 adjuvants were not statistically significantly different for any measure at any time point (all $P > .05$), they were combined for all analyses and compared against placebo values using a *t* test. The results for humoral and cell-mediated data are presented as the geometric mean of each group.

Results

A total of 78 female Holstein calves were enrolled into 1 of the 3 treatment groups: Gr1 n = 24, Gr2 n = 26, and Gr3 n = 28. Enrollment occurred over an 8-week period starting in early September and completed by the end of October 2022.

Seven calves were lost to follow-up during the study. Two calves were incorrectly treated with the first treatment that should have been ineligible for the study. One had a low serum protein, and the second was 11 days of age at first treatment. Two calves died between the first and second vaccination, 1 from group 1 at 13 days of age and 1 from group 2 at 22 days of age; both were reported to have died after diarrhea. These deaths occurred at 5 and 12 days after vaccination, respectively. All 4 of these calves were replaced and were not included in any of the analyzed data. Farm staff did not notify trial personnel of any concerns with vaccine reactions following any treatments.

Table 1 displays the descriptive and calf health statistics for the groups. There were no differences detected between the treatment groups for descriptive or health data. There were no adverse events noted within 1 hour after vaccination. Mild, localized swellings less than 4 cm in diameter were noted at the injection sites in all of the Gr2 and Gr3 calves in the days that followed injection. Group 1 lost 1 calf (4.1%), which was sold at 86 days of age for poor health after pneumonia therapy; Gr2 lost no calves; and Gr3 lost 2 calves (7.1%; $P = .39$). Both calves in Gr3 died after pneumonia therapy at 88 and 90 days of age.

Figure 1 shows the geometric mean with 95% CIs of Ab titers for placebo and vaccine groups throughout the study. Prior to each vaccination ($P = .66$) and at 4 days post second vaccination ($P = .17$), no differences in Ab titers were detected among groups. At 8 days post second vaccination, Ab titers were higher in vaccinates (25,811; 95% CI, 22,783 to 29,241) compared to placebo-treated calves (14,011; 95% CI, 11,502 to 17,069), with a difference of 11,800 ($P < .001$). A detected difference of 13,446 persisted at the final blood collection time point at 61 to 91 days post second vaccination (approx 90 to 120 days of age; $P < .001$).

The highest Ab titers were noted prior to the first vaccination, with a mean titer of 37,099 (95% CI, 30,848 to 44,616) for the placebo calves and a mean titer of 46,049 for the vaccinated calves (95% CI, 39,875 to 53,179; $P = .083$). These high initial titers were likely from colostral transfer of Ab creating nonspecific cross-reactivity within the assay. While there was no evidence of a vaccine effect at the time of the second vaccination, the rapid increase in Ab titer following the second treatment indicated that a substantial anamnestic response did occur following

Table 1—Descriptive statistics of enrollment groups for Holstein calves from a single dairy with adequate levels of passive antibody transfer that were treated with either a saline placebo (Gr1) or 1 of 2 experimental *Salmonella* Dublin siderophore receptor protein (SRP) vaccines with adjuvant A (Gr2) or adjuvant B (Gr3).

	Gr1: placebo (n = 24)	Gr2: *S* Dublin SRP with adjuvant A (n = 26)	Gr3: *S* Dublin SRP with adjuvant B (n = 28)	*P* value
Age at enrollment (d)	7.04 (4–10)	7.0 (4–10)	6.82 (4–10)	.92
Serum protein level (mg/dL)	6.08 (5.5–7.3)	6.33 (5.5–7.3)	6.25 (5.5–7.3)	.191
Age at final blood collection (d)	101.3 (93–119)	105.4 (93–117)	101.4 (91–117)	.136
No. (%) of animals lost (sold/died)	1 (4.1%)	0 (0%)	2 (7.1%)	.39
Age at exit (sold/died)	86 (NA)	NA	89 (88–90)	.33
No. (%) diarrhea cases (first event only)	13 (54.2%)	10 (38.5%)	11 (39.3%)	.46
Age (d) at first diarrhea event	20.1 (6–69)	17.2 (8–48)	12.8 (5–19)	.38
No. (%) pneumonia cases (first event only)	13 (54.2%)	11 (42.3%)	16 (57.1%)	.52
Median (IQR) age (d) at first pneumonia event	45.7 (17–83)	51.1 (7–93)	48.9 (8–95)	.85

Values reported are arithmetic mean and range unless noted. The trial was initiated on September 4, 2022, and concluded on January 15, 2023. Descriptive statistics were compared using ANOVA for the numeric variables and χ^2 or proportion tests for the health event data.

NA = Not applicable.

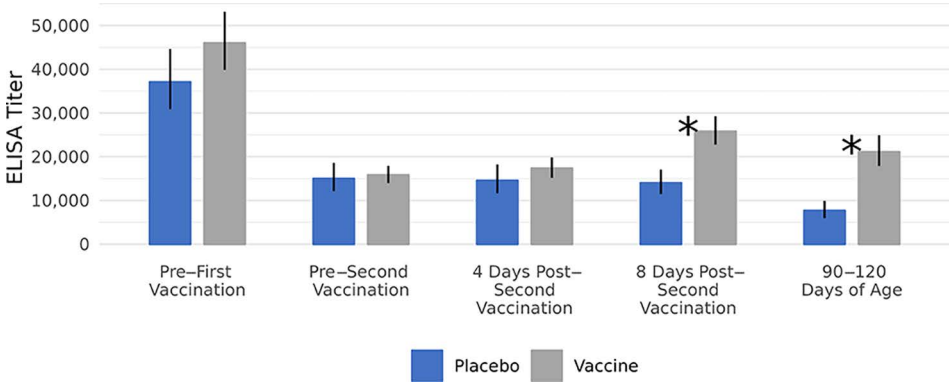

Figure 1—Geometric mean antibody titers (bar) with 95% CIs (black line) for Holstein calves from a single dairy with adequate levels of passive antibody transfer before first and second vaccination, at 4 and 8 days after second vaccination with either experimental *Salmonella* Dublin siderophore receptor protein (SRP) vaccine or saline placebo, and 61 to 91 days after the second vaccination. The trial was initiated on September 4, 2022, and concluded on January 15, 2023. ANOVA was conducted on the log-transformed values to assess adjuvant effects versus placebo-treated calves. *Time points marked with an asterisk were found to be different ($P < .01$).

the second dose of vaccine. These data suggests that *S* Dublin SRP vaccine stimulates an increased Ab titer in comparison to placebo treatment in calves without failure of passive transfer in an *S* Dublin–free herd.

The intra-assay coefficient of variation (CV) for the ELISA assays was 7.03%, and the interassay CV was 12.39% for IL-17. For IFN-γ, the intra-assay CV was 6.13%, and the interassay CV was 11.93%. **Figure 2** shows the geometric mean (95% CIs) IFN-γ ELISpot results and IL-17 and IFN-γ concentration (in ng/mL) following stimulation with 1 µg/mL of SRP antigen. Vaccinates had significantly higher numbers of IFN-γ–producing cells determined by ELISpot at 4 (difference, 93.6 SPU/10^6 cells; $P < .001$) and 8 days (difference, 124.9 SPU/10^6 cells; $P < .001$) after the second dose of vaccine. At 4 days after the second dose of vaccine, the IFN-γ concentration in cell cultures from placebo-treated animals was 69.5 ng/mL (95% CI, 39.2 to 123.3) compared to 106.8 ng/mL (95% CI, 65.6 to 174.1) for vaccinates. No difference was detected between the vaccinate and placebo groups (difference, 37.3; $P = .31$) at day 4, but at day 8 a difference in the concentration of IFN-γ was detected (difference, 71.4 ng/mL; $P = .001$). A difference in concentration of IL-17 vaccinates and placebo-treated calves was apparent at day 4 (difference, 153.9; $P < .001$) and day 8 (difference, 167.8; $P = .009$).

Figure 3 shows the geometric mean (95% CIs) IFN-γ ELISpot results and IL-17 and IFN-γ concentration (in ng/mL) following stimulation with 5 µg/mL of SRP antigen. For the ELISpot analysis, vaccinates had significantly higher numbers of IFN-γ–producing cells determined at 4 (difference, 137.2 SPU/10^6 cells; $P < .001$) and 8 days (difference, 121.2 SPU/10^6 cells; $P < .001$) after the second treatment. Using ELISA, a difference was detected for both IFN-γ and IL-17 concentration at 4 and 8 days after the second treatment. A difference of 109.0 ($P = .036$) and 140.5 ($P < .001$) ng/mL was determined for IFN-γ concentration at 4 and 8 days, respectively, after the second treatment. A difference in concentration of IL-17 was determined between vaccinates and placebo-treated calves at 4 (difference, 200.5 ng/mL; $P < .001$)

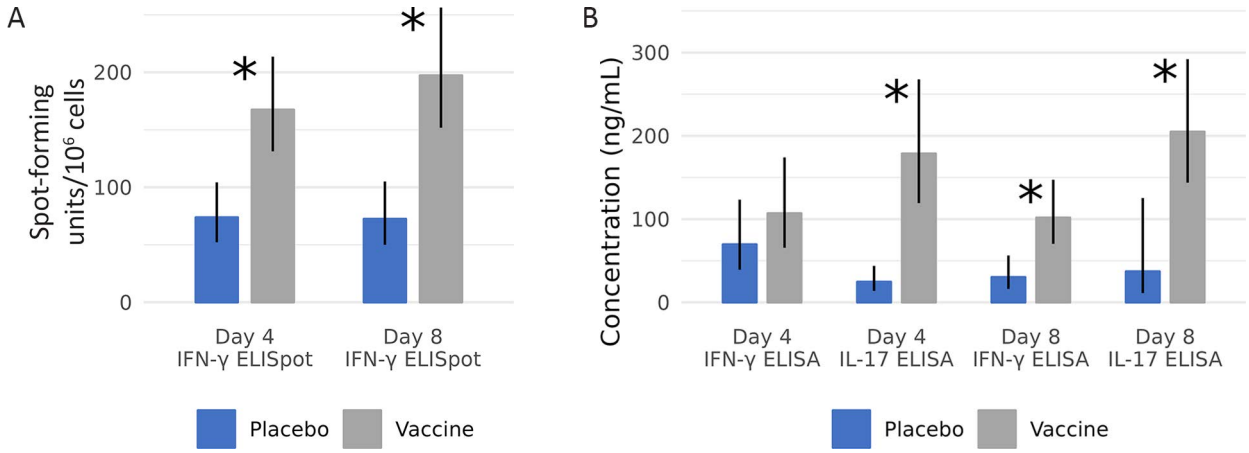

Figure 2—Geometric mean (bar) interferon-γ (IFN-γ) ELISpot** (A) and IL-17 and IFN-γ concentration (in ng/mL; B) determined via ELISA data with 95% CIs (black line) for stimulation with 1 µg/mL *S* Dublin SRP antigen of peripheral blood mononuclear cell (PBMC) samples taken 4 and 8 days after second vaccination with either experimental *S* Dublin SRP vaccine or saline placebo. ANOVA was conducted to assess adjuvant effects versus placebo-treated calves. ELISpot data represent antigen-specific T cells secreting IFN-γ, expressed as spot-forming units/10^6 cells. Numbers of spot-forming units in negative control wells were subtracted to account for background activation. Time points marked with an asterisk were found to be different ($P < .01$).

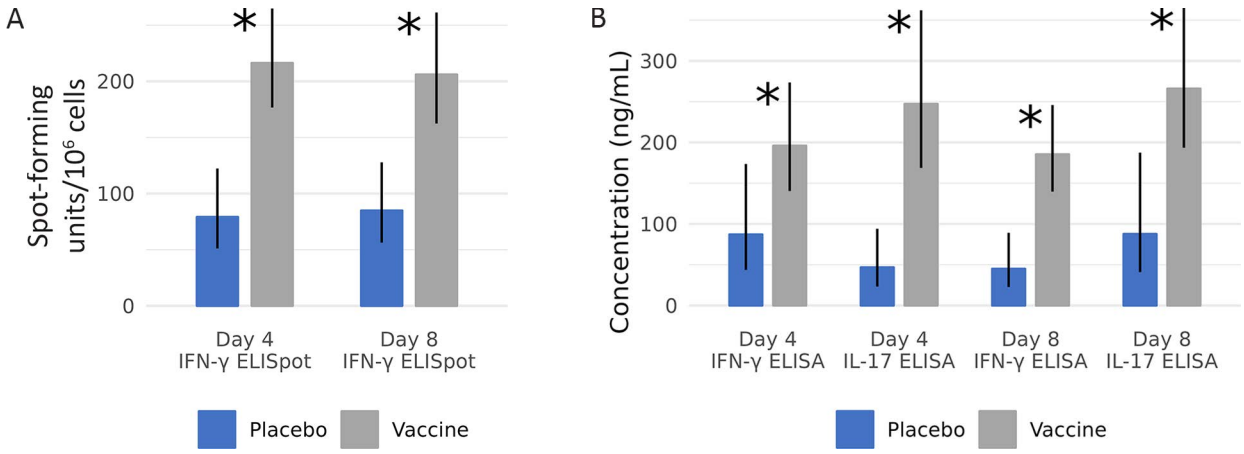

Figure 3—Geometric mean (bar) IFN-γ ELISpot** (A) and IL-17 and IFN-γ concentration (in ng/mL; B) determined via ELISA data with 95% CIs (black line) for stimulation with 5 µg/mL *S* Dublin SRP antigen of PBMC samples taken 4 and 8 days after second vaccination with either experimental *S* Dublin SRP vaccine or saline placebo. ANOVA was conducted to assess adjuvant effects versus placebo-treated calves. ELISpot data represent antigen-specific T cells secreting IFN-γ, expressed as spot-forming units/10^6 cells. Numbers of spot-forming units in negative control wells were subtracted to account for background activation. Time points marked with an asterisk were found to be different ($P < .01$), except for the day 4 IFN-γ ELISA at $P < .036$.

and 8 days (difference, 178.3 ng/mL; $P = .009$) after the second treatment.

All bulk tank milk and individual serum from trial calves collected from 90 to 120 days of age tested with the *Salmonella* ELISA were determined to be negative.

Discussion

These data demonstrate that *S* Dublin–SRP-specific cellular immune responses can be induced in young calves in a short period following vaccination. Studies in other species have demonstrated that Th1 and, to a lesser extent, Th17 responses are vital for the clearance of *Salmonella*. In rodent models, genetic knockout of CD4 T cells or the gene for IFN-γ results in fatal *S* Typhimurium infection.[21] In humans, vaccination with a live, attenuated typhoid vaccine elicited Th1-type CD4 T cells that produced IFN-γ and TNF-α.[22] In mouse models, the presence of IL-17–producing CD4 and γ-δ T cells also enhanced the activity of macrophages and neutrophils, promoting increased bacterial clearance.[23] Pigs vaccinated with a live, attenuated *S* Typhimurium vaccine were shown to mount both Th1 and Th17 responses, which were detectable in peripheral blood.[24] The current study did not involve an *S* Dublin challenge nor was the study terminal; thus, we cannot address the protective role of SRP-specific immune responses against *S* Dublin infection or determine if protective immune response penetrated into the gut and mucosa. However, our data show that young calves can generate SRP-specific Th1- and Th17-type cellular immune responses, which are 1 key factor for protection from systemic *Salmonella* infection.[25] Future studies should focus on determining if SRP vaccination is protective against *S* Dublin infection in young calves as well as dissecting T-cell responses at the site of infection in the gut and mesenteric lymph nodes, which are known to be important sites of defense.

All bulk tank milk and individual serum *Salmonella* ELISA tests were determined to be negative, providing support that the herd enrollment criteria of being free from *S* Dublin was met. However, given the low apparent prevalence in this herd and the diagnostic sensitivity and specificity of the assay, we cannot say for certain that the herd was negative for *S* Dublin. The commercial *Salmonella* ELISA assay can detect O-antigen factors 1, 9, and 12. While it is marketed as an *S* Dublin assay, cross-reaction may occur with other *Salmonella* serotypes.[20] This commercial *Salmonella* ELISA test is readily available at several US veterinary diagnostic laboratories and has been utilized or evaluated in multiple research studies.[1,2,26] In the recent survey of Canadian dairy farms, Perry et al[1] conducted *Salmonella* surveillance on 100 Canadian dairy farms when sampling bulk tanks 2 times and a single blood sample collected from 20 heifers. They reported a 25% herd-level prevalence based on either the bulk tank milk or individual blood tests being reported as positive. However, only 4% of farms were classified as positive based on bulk tank milk results, suggesting that the utility of this test may be suspect to classify herd-level status based on bulk tank milk only. In contrast, a comprehensive evaluation of bulk tank ELISA testing was recently published by another Canadian group.[27] This research group reported a median posterior estimate for sensitivity of 16.3% (95% Bayesian credibility interval, 3.9% to 44.2%) and specificity of 97.5% (95.5% to 99.4%) when using the cutoff employed in the current study (≥ 35% positivity). Their results suggested that classifying a herd as positive based on a single bulk tank survey would require additional complimentary testing; however, a test-negative herd could be classified as true negative with more confidence in the result. Using simulation models, Warnick et al[28] demonstrated that repeated sampling could be used to improve the sensitivity of the

ELISA assay used in the current study. The results of their modeling suggested that in herds with a prevalence of 2%, repeated bulk tank sampling at quarter-year intervals would produce a minimum sensitivity of 92% and minimum specificity of 98%. In a separate Dutch study,[29] herd-level diagnostics for *S* Dublin were evaluated. These authors indicated that bulk tank testing in combination with testing of calves 4 to 6 months of age provided a herd-level sensitivity of 99%.[29] The results of the 2 manuscripts[28,29] were summarized by Nielson et al[30] when describing an early warning system for *S* Dublin in Danish dairy herds. We chose not to test calves with this assay at enrollment due to the potential of maternally derived Ab causing false positive results and lower sensitivity of the assay in calves less than 3 months of age.[30,31] Given the lack of history of *S* Dublin in the herd prior to commencement of the study, the findings of the testing in the current study, and the conclusions of other authors,[27–31] we are as confident as we can be in the negative status of the herd utilized in this study given the available diagnostic capabilities.

Health event monitoring through 90 days of age and comparison of body weight at 100 days of age did not determine differences between groups. There were no postmortem examinations performed on the dead animals. There were no vaccine reactions recorded by trial personnel or farm staff except for swellings at the injection site.

While this study does demonstrate the development of SRP-specific immune responses following vaccination, there are potential weaknesses of the study. The first was not including an antigen-free group for each of the adjuvants. This would have allowed us to tease out the influence of the adjuvant by itself in comparison to the combination of the adjuvant with the antigen. Another potential weakness of the study was our assumption that all IFN-γ measured in the study were from T-responsive lymphocytes as other immune cells are capable of secreting IFN-γ. While there are other immune cells that produce IFN-γ (natural killer cells, monocytes, macrophages), it is predominantly produced by T lymphocytes,[5] particularly in the context of antigen stimulation as in the current study. Additionally, the ELISpot assays utilized in the current study are exquisitely sensitive and widely used by the field to detect antigen-specific T-cell responses of varying cytokine profiles,[32,33] and other studies[34] have demonstrated that the majority of responses detected in the IFN-γ and IL-17 ELISpot assays are derived from CD4 T cells. The final weakness of the study was the inability of trial personnel to monitor the calves on a daily basis to more accurately characterize health events and to rule out any vaccine-related health events. This was due to travel logistics of the farm in relation to research housing of the primary investigators (KPH, CS, and PJG). However, given the fact that no immediate reactions were noted by study personnel or by farm staff, we feel this is minimal in nature.

In conclusion, this study found that vaccination with experimental *S* Dublin SRP vaccines stimulates both cellular and humoral immunity in young calves when vaccinated at 1 and 4 weeks of age. These data demonstrate that *S* Dublin SRP–specific cellular immune responses can be induced in young, colostrum-competent calves in a short period following vaccination. The vaccines only resulted in swellings at the injection site. Further research is needed to determine vaccination efficacy in the face of natural or induced *S* Dublin challenge. These data and those from future studies will help veterinarians develop vaccine protocols against *S* Dublin.

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Disclosures

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