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Immunization with a novel recombinant protein (YidR) reduced the risk of clinical mastitis caused by *Klebsiella* spp. and decreased milk losses and culling risk after *Escherichia coli* infections

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ABSTRACT

The primary objective of this study was to evaluate the protective efficacy of a novel recombinant subunit vaccine containing the protein YidR (rYidR) against clinical mastitis (CM) caused by *Klebsiella* spp. and *Escherichia coli*. Given that *E. coli* infection is known to cause metritis, we also evaluated the effect of rYidR vaccination on the incidence of metritis and conception at the first artificial insemination. Retained placenta and abortion incidence, milk production and composition, and serological responses to specific antigens were also evaluated. In total, 3,107 cows were blocked by parity and randomly allocated into 1 of 3 treatment groups: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and sterile water adjuvanted with aluminum hydroxide (20%; placebo). Vaccinations were performed at the dry-off for cows, and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration was given at 21 ± 3 d after the first injection. Vaccination with rYidR significantly reduced the incidence of CM caused by *Klebsiella* spp. (3.2%) when compared with the placebo (5.1%) group. No difference was observed on risk of *Klebsiella* CM between Kleb-SRP (5.9%) and placebo groups. Cows in the rYidR group that experienced *E. coli* CM had a lower risk of death or culling (12.5%) compared with the Kleb-SRP (27.6%) and placebo groups (27.8%). Furthermore, among cows that developed *E. coli* CM, rYidR-immunized cows produced more milk than did cows in the placebo and Kleb-SRP groups. Regardless of CM occurrence, rYidR-immunized cows tended to have higher milk production up to the eighth month of lactation than cows in the other groups. No significant effect of treatment was observed on the overall incidence of abortion and metritis; however, the risk of retained placenta tended to be lower for the rYidR group (4.7%) compared with the placebo group (6.7%). In addition, primiparous cows in the rYidR group had the highest conception risk at the first artificial insemination (48.3%) compared with the placebo (39.5%) group, and no significant difference was observed when the Kleb-SRP (40.1%) group was compared with the placebo group. Generally, higher antibody serum titers (IgM and IgG) were observed for the immunized groups compared with the placebo. In conclusion, the rYidR vaccine reduced the risk of CM caused by *Klebsiella* spp. and the mortality or culling of cows with *E. coli* infections. Other benefits of the novel vaccine include maintenance of milk production after CM caused by *E. coli*, and higher conception risk at the first service in primiparous cows compared with cows in the placebo and Kleb-SRP groups.

Key words: clinical mastitis, recombinant vaccine, *Klebsiella* spp., coliforms

INTRODUCTION

Mastitis is one of the most important diseases affecting dairy cows and is associated with substantial economic losses to the dairy industry (Halasa et al., 2007; Rollin et al., 2015). For a long time, gram-positive contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae* were the main cause of IMI in dairy herds. However, as a consequence of sustained control programs, the incidence of mastitis caused by contagious bacteria was dramatically reduced over the last 20 yr. In turn, the dairy industry is now facing the challenge of controlling mastitis caused by environmental pathogens, especially coliforms (Ruegg, 2017).

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Escherichia coli and *Klebsiella* spp. are the most frequently isolated coliforms causing clinical mastitis (**CM**) in dairy herds, accounting for approximately 30% of cases in the United States (Oliveira et al., 2013). Both types of bacteria are abundant in cow feces and are the main cause of severe CM, harming the productivity and welfare of dairy cows. *Klebsiella* spp. can induce an aggressive inflammatory response, which is associated with more severe clinical signs and increased risk of death or culling compared with *E. coli* CM (Schukken et al., 2012). The use of preventive practices remains the most effective strategy to reduce the incidence of CM caused by these coliforms, especially because affected cows may not benefit from antimicrobial treatment (Ganda et al., 2016; Fuenzalida and Ruegg, 2019).

Ensuring hygienic practices at the housing facilities and during milking, keeping cows in salubrious conditions, and using effective vaccines are among the strategies to control coliform mastitis in dairy farms (Hogan and Smith, 2003, 2012). Current vaccines available against *E. coli* mastitis are based on the use of *E. coli* bacterins using the J5 strain. Although vaccines composed of J5 bacterins are considered beneficial for reduction of *E. coli* CM severity, no evidence of their ability to prevent *E. coli* CM has been reported (Wilson et al., 2007; Gurjar et al., 2013).

Recently, a vaccine composed of a *Klebsiella pneumoniae* siderophore receptor protein (**SRP**) became commercially available in the United States for prevention of CM caused by *Klebsiella* spp. Thus far, only one study has been published evaluating the efficacy of the vaccine, reporting a significant reduction in the risk of *Klebsiella* CM and total coliform CM (Gorden et al., 2018). Despite the exciting results of that study, only 67 pairs of cows (i.e., vaccinated vs. placebo) were assessed for those outcomes, and only 26 cases of *Klebsiella* CM were diagnosed during the study period. Therefore, further studies conducted in different herds and enrolling a higher number of cows are required to confirm the efficacy of the *K. pneumoniae* SRP vaccine.

To be potentially effective, an anti-*Klebsiella* vaccine must have an antigen expressed by all, or at least by most, of the strains encountered (Babu et al., 2017), but this can be difficult to achieve given the high genetic heterogeneity of *Klebsiella* strains. Based on the existing genomic database of our laboratory, the *yidR* gene was found to be ubiquitous in 308 *Klebsiella* strains from humans and mastitic dairy cows, despite the high genetic diversity among isolates (Yang et al., 2019). In addition, the *yidR* gene was highly conserved within *Klebsiella* spp. and between other gram-negative bacterial species; *E. coli* (96% sequence homology) and *Salmonella enterica* (98.7%; Rodrigues et al., 2020). The YidR protein contains 2 conserved domains that are associated with Tol-dependent translocation of colicins into *E. coli* (Cascales et al., 2007), and it is implicated in the pathogenesis of *Enterobacteriaceae* (Dubuisson et al., 2005). In addition, YidR is a putative ATP/GTP-binding protein which mediates the hyperadherence phenotype (Kroupitski et al., 2013; Megias et al., 2016) and contributes to biofilm formation in some coliforms (Kroupitski et al., 2013). The study performed by Rodrigues et al. (2020) reported the efficacy of recombinant YidR (**rYidR**) vaccine using a mouse lethal challenge model with *K. pneumoniae*; approximately 90% of mice assigned to the rYidR vaccine survived beyond 10 d after challenge, whereas none of the control mice survived past 48 h. Those exciting results indicated that the rYidR subunit vaccine could be a promising immunogenic candidate against naturally occurring CM caused by *Klebsiella* spp. and *E. coli* in dairy cows.

Therefore, the primary objective of this study was to evaluate the protective efficacy of the novel recombinant subunit vaccine containing the protein YidR against CM caused by *Klebsiella* spp. and *E. coli*. We also evaluated the rYidR vaccine's effect on the incidence of abortion, retained placenta and metritis, and risk of death or culling of cows after CM caused by *E. coli* and *Klebsiella* spp., conception risk at first AI, milk production and composition, and serological responses to specific antigens.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the recommendations of The Animal Welfare Act of 1985 (P.L. 99–198). The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University (protocol number 2017–0073).

Study Design

A large double-blinded, placebo-controlled, randomized clinical trial was performed to evaluate the protective efficacy of a recombinant protein subunit vaccine (rYidR) or a commercially available vaccine against bovine CM caused by *Klebsiella* spp. and *E. coli*. Cows received 2 vaccinations during the dry period and were monitored up to 10 mo after calving for the occurrence of CM and performance indicators. Blood samples were collected during the dry period for assessment of antibody responses.

Production of rYidR Vaccine

Protein Production. The rYidR protein was produced in a fed-batch fermentation using a Bioflo and Celligen 310 Fermenter/Bioreactor unit (New Brunswick Scientific, Edison, NJ) equipped with probes for pH, temperature, foam level, and dissolved oxygen. *Escherichia coli* BL21 (DE3) harboring the pET-6xHis/6his-yidR (YidR) vector was cultured in a chemically defined medium (Li and Sha, 2015) with 100 μg/mL of ampicillin, previously prepared in the bioreactor vessel. A detailed description of the batch fermentation parameters and control pre- and postinduction of the protein expression is published elsewhere (Rodrigues et al., 2020).

The induction was carried out when wet cell weight achieved 50%. Temperature and feeding medium were adjusted and isopropyl β-D-1-thiogalactopyranoside was added (1 m*M* final concentration). The induction of protein expression was completed in 24 h. Cells were harvested by centrifuging the fermenter vessels (20,000 × *g*, 3 times, room temperature) and resuspended in 1 × buffer A (50 m*M* NaH_2PO_4; 300 m*M* NaCl, pH 7.4). Next, cells were lysed by sonication (5 min-pulses for 5 times, 80% amplitude), the sonicated cells were centrifuged (17,000 × *g* for 30 min, 4°C), and the supernatant obtained was stored at 4°C for purification.

Protein Purification. The automated purifications were performed based on immobilized metal affinity chromatography using a custom-packed column of TALON Metal Affinity Resin (Takara Bio USA Inc., Mountain View, CA), followed by desalting carried out on 1,200-mL Sephadex G-25 column (GE Healthcare, Chicago, IL). The latter processes were described in detail in a previous study (Rodrigues et al., 2020). Purification and desalting were conducted using an ÄKTA Pure chromatography system (GE Healthcare) controlled by Unicorn 7 software (GE Healthcare).

Vaccine Preparation. The purified protein was sterilized by filtration using a 0.22 μm vacuum filter system (Millipore Sigma, Burlington, MA), quantified using a Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA), and stored in 50% glycerol at −20°C after verification of endotoxin content (Rodrigues et al., 2020). The vaccine contained 1,000 ng/mL of YidR protein and 20% of vaccine adjuvant (aluminum hydroxide gel, Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA).

Farm and Cow Selection

The study was conducted on a large commercial dairy farm located in Cayuga County near Ithaca, New York. The selected farm milked approximately 4,100 Holstein cows thrice daily in a 100-stall rotary milking parlor with integrated milk meters that recorded individual milk production at every milking (DeLaval, Tumba, Sweden). Dry and milking cows were housed in freestall barns, with concrete stalls covered with mattresses and bedded with manure solids. The herd had an average bulk milk SCC of 135,330 cells/mL and daily milk production of 40.4 kg/cow at the beginning of the study. The monthly mean incidence risk of CM (regardless of mastitis-causing pathogen) during the year before the start of the study (December 2017 to November 2018) was 12.3%. During the same period, the cumulative incidence of CM caused by *Klebsiella* spp. was 20.2% (monthly average of 1.7%), whereas the cumulative incidence of CM caused by *E. coli* was 20.7% (monthly average of 1.7%).

All animals were subjected to the same immunization protocol before and during the study period according to the farm's existing vaccination schedule. At 200 d of pregnancy, they were immunized with Triangle 9 (Boehringer Ingelheim Vetmedica Inc., Duluth, GA) and Covexin (Merck Animal Health, Summit, NJ). At 250, and again at 264 d pregnant they were immunized with J-Vac (Merial, Duluth, GA) and Scourguard (Zoetis, Florham Park, NJ). Finally, at 35 DIM, they were immunized with Vista 5 SQ (Merck Animal Health) and J-Vac, and at the first pregnancy diagnosis date, they received a fourth dose of J-Vac. Therefore, all animals in the study were scheduled to receive a total of 4 injections of an *E. coli*-J5 mastitis vaccine (J-Vac). Trained farm personnel administered all nonexperimental vaccines.

Animals were blocked by parity group (nulliparous, primiparous, and multiparous) and randomly allocated to one of the experimental treatments using the RAND function of Excel software (2016, Microsoft Office Corporation, Redmond, WA). Cows with ≥1 lactations were enrolled within 3 d after drying off (approximately 60 ± 3 d before calving), whereas heifers were enrolled at 223 ± 3 d pregnant. The vaccination worksheets were prepared weekly, on the day before vaccination, based on reports extracted from the Dairy Comp 305 (**DC305**) software (Ag Valley Agricultural Software, Tulare, CA).

Experimental Treatments and Vaccination Protocols

Vaccines and placebo preparations were stored in identical amber containers and labeled with the letters A, B, and C. Treatments were undisclosed to the people administering the treatment, examining the cows for disease and collecting clinical data, or to any farm worker. The code was only broken at the end of the follow-up period, after all data were analyzed.

Based on the aforementioned criteria, cows recently dried off and pre-fresh heifers (223 ± 3 d pregnant) were randomly assigned to one of the following treatments: injection with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (**Kleb-SRP**; KlebVax, Epitopix, Willmar, MN); injection with 2 mL of the experimental recombinant subunit vaccine containing the protein YidR (rYidR; 2,000 ng of rYidR per injection); and injection with 2 mL of the adjuvant used in the latter vaccine (placebo; aluminum hydroxide, Alhydrogel adjuvant 2%, InvivoGen). A booster was given to all animals 21 ± 3 d after the first injection (approximately 244 ± 3 d of pregnancy). All injections were given subcutaneously in the neck region using automatic self-filling syringes (Allflex, Dallas, TX) and one 18 G × 1 needle per animal. Local reactions were visually monitored after each vaccination through the study period.

Blood Sampling and Antibody Responses

Three blood samples were collected from approximately 14% of animals assigned to each group at the first and second treatment inoculations, and at 14 d after the second inoculation. Cows submitted for blood sample collection were randomly selected based on their ascending numerical order (i.e., first animals were selected) determined by the RAND function of the Excel software using the previously described parity-blocked list created on a weekly basis. Sample collection was performed via coccygeal vein/artery puncture into a 10-mL Vacutainer tube without anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ) for serum separation. All blood samples were transported to the laboratory on ice and centrifuged at 2,000 × *g* for 15 min at 4°C. Serum was harvested and frozen at −80°C until assayed.

The relative quantification of IgG and IgM was performed using the serum samples collected at the 3 aforementioned time points. Enzyme-linked immunosorbent assay microplates were coated with antigen diluted in 1 × PBS (pH 7.4). Microplates were coated with 1 × PBS containing the following antigens: (1) 0.25 mg/mL of purified rYidR protein, (2) 0.25 mg/mL of Kleb-SRP, (3) 10^6 cfu/mL of *Klebsiella pneumoniae* (strain #4798 isolated from bovine CM), or (4) 10^6 cfu/mL *Escherichia coli* (ATCC 25922). The only exception was performed for the IgM assay using the purified rYidR as antigen, in which 0.5 mg/mL of the recombinant protein was used. Binding of antigen to microplate wells was carried out overnight at 4°C. Next, bovine serum samples were thawed, mixed and diluted before adding to the ELISA plates. Serum samples were diluted to 1:500, 1:100, 1:500, and 1:500 for anti-rYidR, anti-KlebVax, anti-*K. pneumoniae*, and anti-*E. coli*, respectively, for the IgG assay. For the IgM assay, serum samples were diluted to 1:500, 1:100, 1:1,000, and 1:1,000 for anti-rYidR, anti-KlebVax, anti-*K. pneumoniae*, and anti-*E. coli*, respectively. The diluted samples were added to the plates and incubated overnight at 4°C. Anti-bovine IgG:HRP (Sigma-Aldrich) diluted to 1:20,000 and anti-bovine IgM:HRP (Thermo Fisher, Waltham, MA) diluted to 1:10,000 were transferred to the respective plates and incubated for 45 min at room temperature. Postincubation, the wells were washed 3 times with washing solution (1 × PBS with 0.1% Tween-20). Following secondary antibody binding and washing, the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) was added, followed by 1 *M* HCl. The resulting yellow end product was then read at 450 nm using an ELISA plate reader (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). The optical density detected was proportional to the amount of primary antibody bound to the antigen-binding sites on the wells. For assays using whole antigen (*K. pneumoniae* and *E. coli*), the bacterial cells were treated as previously described by Borowski et al. (1984). The commercial vaccine (Kleb-SRP) was submitted to a buffer exchanging procedure for exclusion of additives, adjuvants, or both; thus, the ELISA targeted the vaccine's protein content.

Monitoring and Follow-Up

Within a week of the first dose administration, all enrolled animals were entered into the farm's DC305 database (Ag Valley Agricultural Software, Tulare, CA). Occurrence of abortion and diseases such as CM, metritis, and retained placenta, as well as indicators of cow performance (i.e., milk production, composition and SCC, and conception at first service) were assessed based on DC305 records. All diseases were diagnosed and treated by trained farm personnel, which were not aware of the study treatments. The occurrence of disease was followed from calving until the date of drying off, culling, or death, or at the end of the study. Performance indicators at the cow level, such as milk production, linear score of SCC (**LSSCC**) and milk composition (fat and protein content) were assessed based on monthly DHIA test results from calving up to the eighth test date performed for each cow. The risk of conception at the first AI was evaluated as an indicator of reproductive performance, which was based on farm records.

Disease Definitions

Clinical mastitis was defined as the presence of abnormal milk, such as watery appearance or flakes and clots in milk during forestripping detected at each milking. Once diagnosed in the milking parlor, the cow was immediately sorted, and a trained farm employee collected a milk sample from the infected quarter(s) after disinfection of the teat end(s) using cotton pads soaked with 70% alcohol. Once collected, the milk sample was refrigerated at the farm (≅4°C) and information about the case (i.e., cow's ID, affected quarter, and date of identification) was recorded into the DC305. Every 24 h, a courier from the bacteriology laboratory (Quality Milk Production Services Laboratory at Cornell University) traveled to the farm to gather the samples along with an electronic form containing the list of cows with CM identified since the previous visit. Microbiological culture techniques were performed following the National Mastitis Council guidelines (NMC, 2017). Within 48 h after pick-up, the preliminary culture results were transferred from the Quality Milk Production Services Laboratory to the farm's computer via DC305. Based on culture results, only cows with nonsevere CM caused by gram-positive bacteria (except those caused by *Staphylococcus aureus* and *Trueperella pyogenes*) received 3 intramammary infusions (once a day) with hetacillin potassium (equivalent to 62.5 mg of ampicillin; PolyMast, Boehringer Ingelheim Animal Health, Duluth, GA). Cows identified with systemic clinical signs did not receive intramammary antimicrobials. Instead, they were administered with intravenous supportive therapy (750 mg of flunixin meglumine, 1,000 mL of 7.5% hypertonic saline, and 500 mL of 23% calcium gluconate solution) to relieve systemic clinical signs of the disease.

Retained placenta was defined as cows that failed to release the fetal membranes within 24 h after calving (Kelton et al., 1998). Metritis was defined as the presence of fetid, watery, red-brown uterine discharge accompanied with fever (rectal temperature > 39.5°C). Abortion was defined as the loss of pregnancy from enrollment to parturition based on visual signs of fetal death (i.e., spontaneous expulsion of a dead fetus), return of the cow to estrus, or both. Cow death or culling for any reason or because of mastitis (overall and by pathogen) was assessed throughout the follow-up period.

Data Analysis

Descriptive statistical analyses were carried out with JMP PRO 14 (SAS Institute Inc., Cary, NC) using the ANOVA function for continuous data, and chi-squared and Fisher's tests for categorical data.

For analyses of repeated measurements, such as serum antibody responses (IgG and IgM), LSSCC, and milk yield and composition, we used general mixed linear models with the MIXED procedure of SAS (version 9.4; SAS/STAT, SAS Institute Inc.). The independent variables offered to the models were treatment, time, parity (primiparous or multiparous), presence of nonfunctional mammary quarter (yes or no) at calving, and LSSCC at the dry-off. In addition, biologically plausible 2-way interaction terms between independent variables were added to the models. Two scenarios were used to compare milk yield and LSSCC between treatments. The first scenario accounted for the overall results of those variables based on the first 8 DHIA test results performed monthly after calving; this analysis was performed regardless of CM occurrence and mastitis-causing pathogens. The second scenario considered only cases in which the cows had CM caused by *E. coli* or *Klebsiella* spp. and factored in the DHIA results obtained before and after the disease diagnosis. For the latter models, 3 time points relative to CM were assessed based on bi-monthly averages of milk production and LSSCC. Time point −1 was generated based on 2 test dates performed before CM diagnosis, whereas the time points 1 and 2 corresponded to the averages of the first and second, and the third and fourth test dates after CM identification, respectively. This approach for data analysis was performed to reduce the potential bias associated with the time range between the occurrence of CM and the first DHIA test after diagnosis, which could vary from a few days to up to weeks.

Data analyses of dichotomized outcomes such as occurrence of CM (overall and caused by *Klebsiella* spp. or *E. coli*), abort, retained placenta, metritis, death or culling, and conception at the first insemination were performed by using multivariate logistic regression models and the binary distribution of the GLIMMIX procedure (SAS version 9.4). Models were created to evaluate treatment outcomes considering all enrolled cases, and using the data stratified according to the cow parity after calving (primiparous and multiparous). The risk of mastitis was compared between experimental groups considering the overall frequency of CM, and according to the mastitis-causing pathogen (*Klebsiella* spp. or *E. coli*). For assessment of mastitis occurrence, the independent variables offered to the models were treatment, parity (primiparous or multiparous), and interaction between treatment and parity. The number of CM cases in the previous lactation, presence of afunctional quarters (yes or no), and linear score of SCC at the dry-off were also added to the models evaluating

Table 1. Descriptive statistics of pretreatment cow-level characteristics (mean; SD in parentheses) and distribution of cows according to intramammary treatment group

	Treatment[1]			
Item	rYidR	Kleb-SRP	Placebo	*P*-value
Enrolled animals	1,036	1,026	1,045	0.86
Pre-fresh heifers	373	367	380	0.99
First lactation	279	268	284	
Second lactation	172	175	173	
Third or more lactation	212	216	208	
Days carried calf	221 (12)	221 (14)	221 (13)	0.63
Age in days	1,136 (557)	1,135 (540)	1,133 (557)	0.99
DIM	322 (52)	321 (58)	322 (58)	0.92
LSSCC[2]	3.1 (1.5)	3.2 (1.6)	3.2 (1.5)	0.81
Milk yield (kg/d)	62 (20)	61 (21)	61 (19)	0.32

[1]rYidR = 2 injections with 2 mL of the experimental recombinant subunit vaccine containing the YidR protein; Kleb-SRP = 2 injections with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (KlebVax, Epitopix, Willmar, MN); placebo = 2 injections with 2 mL of the aluminum hydroxide adjuvant (Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA). The vaccinations were performed at the dry-off (approximately 60 d before the expected calving date) for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration (booster) was performed in all animals 21 ± 3 d after the first inoculation.

[2]LSSCC = linear score of somatic cell count calculated as follows: LSSCC = $\log_2$ (SCC/100) + 3.

multiparous cows. Models evaluating the incidence of other diseases or clinical events (i.e., abortion, retained placenta, conception risk at the first insemination, culling or death) included the effects of treatment, parity, and interaction between treatment and parity. The effect of treatments on risk of cow death or culling was evaluated regardless of the event reason, and considering only cases in which the cow was culled or died because of mastitis (caused by any pathogen, and after infections caused by *E. coli* or *Klebsiella* spp.). Dunnett's significance test was used to compare cows in the rYidR or Kleb-SRP groups with cows in the placebo group.

For all data analyses used to assess repeated measures and dichotomized outcomes, final multivariable models were reached after performing a manual backward stepwise elimination procedure. After each run, variables and their respective interaction terms with the highest *P*-value were excluded from the model until all variables had $P \leq 0.10$. Potential confounders were monitored by the change in the coefficient of a variable after removing another variable from the model. Variables were considered statistically significant when a *P*-value ≤ 0.05 was detected. A tendency to significance was considered if the *P*-value was between 0.05 and 0.10.

For description of postcalving outcomes, animals previously vaccinated as heifers are described as "primiparous cows," whereas animals that experienced one or more lactations at vaccination are described as "multiparous cows."

RESULTS

Descriptive Data

The vaccination trial was conducted from December 2018 to August 2019. Cows were monitored from enrollment until the day of drying off, culling or death, or until the end of data collection (December 2019). In total, 3,107 animals were enrolled in the study; of these, 1,036 were assigned to the rYidR arm, 1,026 to the Kleb-SRP arm, and 1,045 to the placebo arm (Table 1). Pre-fresh heifers accounted for 1,120 enrolled animals and 1,987 cows with ≥1 lactation comprised the remainder of the study cohort. No significant differences in the distribution of animals between groups were detected according to parity and days carried calf at enrollment, age in days and DIM at drying off (cows), and LSSCC and milk yield before drying off based on the test date results (Table 1).

In total, 2,095 cases of CM from 1,121 cows were recorded during the study period. Of these, 659 (31.5%) were identified in the rYidR group, 714 (34.1%) in the Kleb-SRP group, and 722 (34.5%) in the placebo group. Gram-positive pathogens were isolated from 797 (38.0%) CM cases, gram-negative bacteria accounted for 619 (29.5%) CM cases, and 592 (28.3%) of the milk cultures had no growth. In addition, cultures from 87 CM cases were either contaminated (≥3 bacterial colonies with distinct morphological features; n = 19), mixed (i.e., colonies presenting 2 distinct morphological characteristics, n = 15), or diagnosed with nonbacterial

organisms (e.g., yeast and *Prototheca* spp.) or other microorganisms not identified using the routine methods (n = 53). Overall, *E. coli* and *Klebsiella* spp. were isolated from 338 (16.1%) and 242 (11.6%) cases of CM, respectively, during the follow-up period.

One heifer at 618 d of life and 226 d pregnant died after vaccination with Kleb-SRP. The heifer presented with severe swelling the size of a basketball at the injection site (neck) and intense breathing difficulty within 12 h of injection. After attempting to alleviate the symptoms using supportive therapy, the heifer was euthanized due to suspicion of anaphylactic shock, although a necropsy was not performed to confirm the cause of death.

Effect of Vaccination on CM Risk

Overall (regardless of mastitis-causing pathogen), no effect of treatment was observed on risk of CM ($P = 0.19$; Table 2). However, the overall CM in primiparous cows tended to be lower in the rYidR group (20.6%) compared with placebo group (27.1%; Figure 1). When evaluating the effect of immunization on CM caused by *Klebsiella* spp., the rYidR group had the lowest risk of disease, regardless of parity (Table 2). In addition, the risk of *Klebsiella* CM was lower for multiparous cows in the rYidR group than placebo group (Figure 1). Unexpectedly, no difference of *Klebsiella* CM was observed between the Kleb-SRP and placebo groups. Likewise, no differences between experimental groups were observed on risk of CM caused by *E. coli* (Table 2; Figure 1).

Effect of Vaccination on Incidence of Abortion, Retained Placenta, and Metritis

In total, 91 cases (2.9%) of abortion were recorded from enrollment until parturition and no significant difference was observed between treatment groups (Table 3). Retained placenta was recorded for 202 (6.5%) cows enrolled in the study. A tendency of treatment effect ($P = 0.09$) was observed between treatments, in which rYidR-immunized cows (4.7%) tended to have lower incidence of retained placenta than cows in the placebo group (6.7%). Metritis was detected in 347 (11.2%) cows and no differences were observed when the vaccine groups were compared with placebo. The interaction terms of abortion, retained placenta, and metritis with parity (primiparous or multiparous) were not significant (Table 3).

Effect of Vaccination on Risk of Death, Culling, or Both

Regardless of mastitis occurrence, there was no effect of treatments on risk of death or culling (Table 4). In total, 578 cows enrolled in the study were culled or died during the follow-up period; of these, 164 (28.4%) events occurred in primiparous cows and 414 (71.6%) in multiparous. Likewise, no difference between groups was observed on risk of culling/death due to mastitis, regardless of pathogen ($P = 0.18$), or when culling/death occurred after *Klebsiella* spp. CM ($P = 0.25$). However, rYidR-immunized cows had a significantly lower risk of culling or death following diagnosis of *E. coli* CM (12.5%) compared with cows assigned to

Table 2. Effects of vaccination on risk of clinical mastitis (overall and at the pathogen level) diagnosed during lactation of 3,107 Holstein cows enrolled in the study[1]

	Adjusted incidence[2]			Odds ratio (95% CI)		*P*-value		
Item	Placebo[3]	rYidR[4]	Kleb-SRP[5]	rYidR	Kleb-SRP	TRT	Parity	TRT × parity
Overall	36.8	34.4	38.1	0.84 (0.66, 1.06)	0.96 (0.75, 1.22)	0.19	<0.0001	0.054
Klebsiella spp.	5.1[a]	3.2[b]	4.9[a]	0.62 (0.41, 0.96)	0.96 (0.66, 1.43)	0.03	<0.0001	0.93
Escherichia coli	7.8	6.5	6.7	0.81 (0.57, 1.16)	0.84 (0.60, 1.20)	0.37	<0.0001	0.80

[a,b]Different lowercase superscripts within a row indicate significant differences between treatments ($P < 0.05$).

[1]All vaccinations were performed at the dry-off (approximately 60 d before the expected calving date) for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration (booster) was performed in all animals 21 ± 3 d after the first inoculation.

[2]Adjusted incidence of clinical mastitis based on LSM multiplied by 100. The independent variables offered to the multivariate logistic regression models were treatment, parity, occurrence of retained placenta or metritis, and biologically plausible interactions. Dunnett's significance test was used to compare cows immunized with rYidR or Kleb-SRP with cows in the placebo group.

[3]Placebo = 2 injections with 2 mL of the aluminum hydroxide adjuvant (Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA).

[4]rYidR = 2 injections with 2 mL of the experimental recombinant subunit vaccine containing the YidR protein.

[5]Kleb-SRP = 2 injections with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (KlebVax, Epitopix, Willmar, MN).

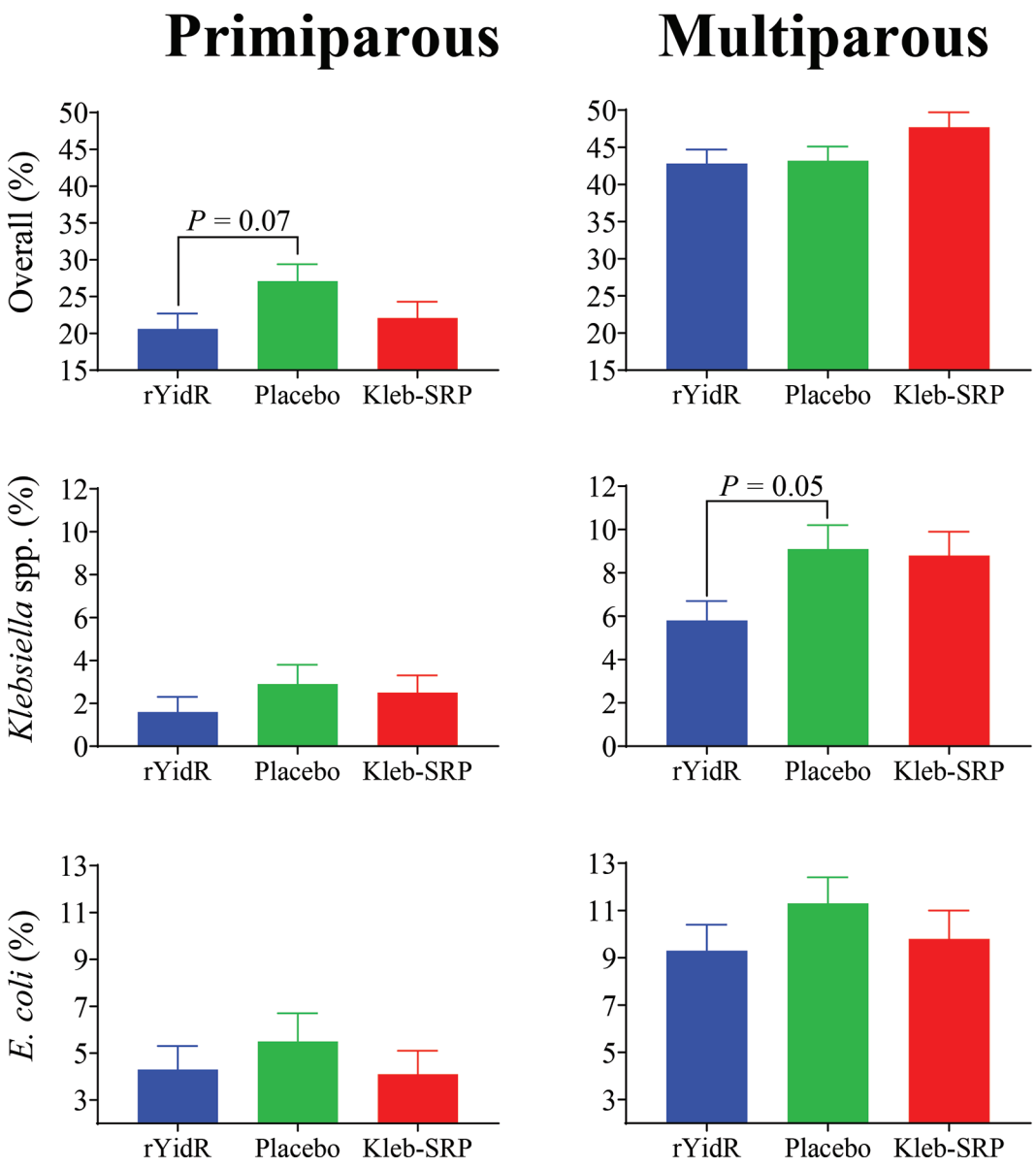

Figure 1. Overall and pathogen-level risk of clinical mastitis estimated for primiparous (first lactation) and multiparous (≥2 lactations) cows randomly assigned to 1 of 3 vaccination protocols: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and aluminum hydroxide adjuvant (placebo). The vaccinations (2 mL/injection) were administered at the dry-off for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second inoculation was given to all animals 21 ± 3 d after the first inoculation. The independent variables offered to the regression models are described in Table 1. Results are presented as LSM ± SEM. $P \leq 0.05$.

the placebo group (27.8%, $P = 0.01$). No difference in the risk of death or culling because of *E. coli* CM was observed between cows in the Kleb-SRP and placebo groups (Table 4).

Effect of Vaccination on Conception Risk at First AI

Conception at first AI was assessed as an indicator of reproductive performance between groups after calving. By the end of the data collection, 2,657 enrolled cows experienced at least one AI after calving and had results of pregnancy diagnosis. Overall (regardless of parity), rYidR-immunized cows tended to have a higher conception risk at the first service (42.0%) than cows in the placebo group (37.4%; Table 5). Although the conception risk at the first AI was statistically similar among multiparous cows, primiparous cows in the rYidR group had the highest conception risk at the first

Table 3. Effects of vaccination on postcalving risk of abortion, retained placenta, and metritis of 3,107 Holstein dairy cows enrolled in the study[1]

	Adjusted incidence[2]			Odds ratio (95% CI)		*P*-value		
Item	Placebo[3]	rYidR[4]	Kleb-SRP[5]	rYidR	Kleb-SRP	TRT	Parity	TRT × parity
Abortion	3.4	2.3	2.7	0.69 (0.37, 1.28)	0.80 (0.43, 1.48)	0.39	0.32	0.69
Retained placenta	6.7[A]	4.7[B]	7.0[A]	0.68 (0.43, 1.08)	1.05 (0.70, 1.59)	0.09	0.02	0.64
Metritis	11.6	10.3	13.0	0.65 (0.63, 1.21)	1.14 (0.84, 1.55)	0.18	<0.0001	0.79

[A,B]Different uppercase superscripts within a row indicate a trend between treatments ($0.05 < P \leq 0.10$).

[1]All vaccinations were performed at the dry-off (approximately 60 d before the expected calving date) for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration (booster) was performed in all animals 21 ± 3 d after the first inoculation.

[2]Adjusted incidence of disease based on LSM multiplied by 100. Models evaluating the risk of abortion included the variables treatment, parity, and the interaction between treatment and parity. Models evaluating the frequencies of retained placenta and metritis included treatment, parity, calf status at calving (dead, alive, or twins), and retained placenta occurrence (only for metritis models) as independent variables. Biologically plausible interactions were assessed in all models. Dunnett's significance test was used to compare cows immunized with rYidR or Kleb-SRP with cows in the placebo group.

[3]Placebo = 2 injections with 2 mL of the aluminum hydroxide adjuvant (Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA).

[4]rYidR = 2 injections with 2 mL of the experimental recombinant subunit vaccine containing the YidR protein.

[5]Kleb-SRP = 2 injections with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (KlebVax, Epitopix, Willmar, MN).

AI (48.3%) compared with primiparous cows in the placebo group (39.5%). No significant difference was observed in the conception risk at the first AI when primiparous cows in the Kleb-SRP group (40.1%) were compared with primiparous cows in the placebo group (Table 5).

Milk Yield, Composition, and LSSCC

In total, 17,260 records for each performance outcome (i.e., milk production, LSSCC, and fat and total protein content) were assessed based on DHIA test dates from calving to the eighth month of cow lactation. Not all cows had complete data because of events such as culling, death, missing tests, or end of study. Throughout the aforementioned follow-up period, the overall average (±SD) milk production was 45.0 ± 10.3 kg, while the average LSSCC was 2.2 ± 2.0. In addition, the milk of the enrolled cows had an average fat content of 4.1% (0.7) and total protein content of 3.0% (0.3).

Results comparing the overall milk production, composition, and LSSCC between treatments are presented in Figure 2. There was a tendency for the effect of treatment on milk production ($P = 0.09$) during the first 8 test dates after calving (Figure 2-A). No effect of treatment was observed on overall LSSCC (Figure 2B), milk fat content (Figure 2C), and total protein content (Figure 2D).

Table 4. Effects of vaccination on postcalving risk of death or culling of 3,107 Holstein dairy cows enrolled in the study[1]

	Adjusted incidence[2]			Odds ratio (95% CI)		*P*-value		
Item	Placebo[3]	rYidR[4]	Kleb-SRP[5]	rYidR	Kleb-SRP	TRT	Parity	TRT × parity
Overall[6]	15.9	18.3	18.2	1.19 (0.91, 1.57)	1.18 (0.89, 1.58)	0.30	<0.0001	0.12
Due to mastitis[7]	5.3	4.1	5.6	0.77 (0.51, 1.16)	1.06 (0.72, 1.55)	0.18	<0.0001	0.31
Klebsiella spp.	21.8	33.7	31.3	1.82 (0.75, 4.40)	1.64 (0.73, 3.64)	0.25	0.06	0.74
Escherichia coli	27.8[a]	12.5[b]	27.6[a]	0.37 (0.17, 0.83)	0.99 (0.49, 2.02)	0.01	0.003	0.70

[a,b]Different lowercase superscripts within a row indicate significant differences between treatments ($P < 0.05$).

[1]All vaccinations were performed at the dry-off (approximately 60 d before the expected calving date) for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration (booster) was performed in all animals 21 ± 3 d after the first inoculation.

[2]Adjusted risk of culling or death based on LSM multiplied by 100. Models added the variables treatment, parity, calf status at calving, and occurrence of diseases (only in the overall assessment). Biologically plausible interactions were assessed in all models. Dunnett's significance test was used to compare cows immunized with rYidR or Kleb-SRP with cows in the placebo group.

[3]Placebo = 2 injections with 2 mL of the aluminum hydroxide adjuvant (Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA).

[4]rYidR = 2 injections with 2 mL of the experimental recombinant subunit vaccine containing the YidR protein.

[5]Kleb-SRP = 2 injections with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (KlebVax, Epitopix, Willmar, MN).

[6]Effect of treatments on risk of culling or death, regardless of the reason of event.

[7]Effect of treatments on risk of culling or death of cows due to mastitis (overall and after infections caused by *Klebsiella* spp. or *E. coli*).

Milk yield and LSSCC were also evaluated relative to the occurrence of CM caused by *E. coli* or *Klebsiella* spp. (Figure 3). The rYidR-immunized cows that experienced *E. coli* CM produced more milk at time point 2 (i.e., average milk production of the third and fourth test dates after CM diagnosis) than cows in the placebo and Kleb-SRP groups (Figure 3A). In addition, rYidR-immunized cows had higher LSSCC at time point 1 (i.e., average of the first and second test dates after CM diagnosis) than cows vaccinated with Kleb-SRP (Figure 3D). No other differences between treatments were observed in terms of milk yield and LSSCC relative to CM caused by *E. coli* and *Klebsiella* spp.

Serological Responses to Vaccination

The results of vaccination on ELISA-detected serum IgG and IgM against several antigens are presented in Figures 4 and 5, respectively. As expected, rYidR vaccine induced the highest increase of serum IgG and IgM titers when rYidR was used as the antigen (Figures 4A and 5A, respectively). Likewise, the serum from cows assigned to the Kleb-SRP group had the highest IgG and IgM titers when the Kleb-SRP vaccine was used as antigen (Figures 4B and 5B, respectively).

No treatment effect was observed on IgG and IgM titers when *E. coli* was used as the antigen (Figure 4C and 5C, respectively). However, when *K. pneumoniae* was the antigen, IgM titers were higher for Kleb-SRP at 21 and 35 d after enrollment compared with the placebo group (Figure 5D). Furthermore, rYidR-immunized cows had higher IgM titers than cows in the placebo group at 35 d after enrollment (Figure 5D).

DISCUSSION

Escherichia coli and *Klebsiella* spp. are the most prevalent gram-negative pathogens causing CM in dairy herds, and the use of organic bedding materials such as recycled dried manure solids may be associated with increased incidence of CM caused by these pathogens (Hogan and Smith, 2012; Rowbotham and Ruegg, 2016). Throughout the present study, *E. coli* and *Klebsiella* spp. accounted for approximately 28% of all CM cases, notwithstanding the farm's strict vaccination protocol using a licensed J5 vaccine. These results reinforce the difficulty in preventing CM caused by coliforms in dairy farms, especially in those with intensive housing systems. Nevertheless, our study showed that the use of a new vaccine containing the recombinant YidR protein had a protective effect against *Klebsiella* spp. causing CM. Cows immunized with rYidR had a 37% reduction in *Klebsiella* CM risk compared with the placebo group.

Before conducting the study, our research group used a murine challenge model to evaluate the protective efficacy of the rYidR vaccine against a pathogenic *K. pneumoniae* strain isolated from bovine CM (Rodrigues et al., 2020). Two immunizations (7 d apart) using rYidR were given to the experimental group, whereas the control group received 2 injections of PBS. None of the nonimmunized mice survived beyond 48 h postchallenge; however, a remarkable survival rate (92.3%) was observed in the rYidR group, indicating the strong protective effect of the novel vaccine against *K. pneumoniae*. Moreover, rYidR-immunized mice had higher antibody induction (anti-rYidR IgG) and lower

Table 5. Effects of vaccination on postcalving conception risk at the first AI of 2,657 Holstein dairy cows (978 primiparous and 1,679 multiparous) enrolled in the study[1]

	Adjusted risk[2]			Odds ratio (95% CI)		*P*-value		
Item	Placebo[3]	rYidR[4]	Kleb-SRP[5]	rYidR	Kleb-SRP	TRT	Parity	TRT × parity
Overall	37.4[B]	42.0[A]	37.2[B]	1.22 (0.99, 1.48)	0.99 (0.78, 1.24)	0.08	<0.001	0.24
Primiparous	39.5[b]	48.3[a]	40.1[b]	1.43 (1.01, 2.02)	1.03 (0.72, 1.46)	0.05	—	—
Multiparous	35.2	36.1	34.5	1.04 (0.79, 1.37)	0.97 (0.73, 1.28)	0.85	—	—

[a,b]Different lowercase superscripts within a row indicate significant differences between treatments ($P < 0.05$).

[A,B]Different uppercase superscripts within a row indicate a trend between treatments ($0.05 < P \leq 0.10$).

[1]All vaccinations were performed at the dry-off (approximately 60 d before the expected calving date) for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second administration (booster) was performed in all animals 21 ± 3 d after the first inoculation.

[2]Adjusted risk of conception at the first AI, based on LSM multiplied by 100. Models added the variables treatment, parity, calf status at calving, and occurrence of diseases (only in the overall assessment). Biologically plausible interactions were assessed in all models. Dunnett's significance test was used to compare cows immunized with rYidR or Kleb-SRP with cows in the placebo group.

[3]Placebo = 2 injections with 2 mL of the aluminum hydroxide adjuvant (Alhydrogel adjuvant 2%, InvivoGen, San Diego, CA).

[4]rYidR = 2 injections with 2 mL of the experimental recombinant subunit vaccine containing the YidR protein.

[5]Kleb-SRP = 2 injections with 2 mL of a commercially available vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (KlebVax, Epitopix, Willmar, MN).

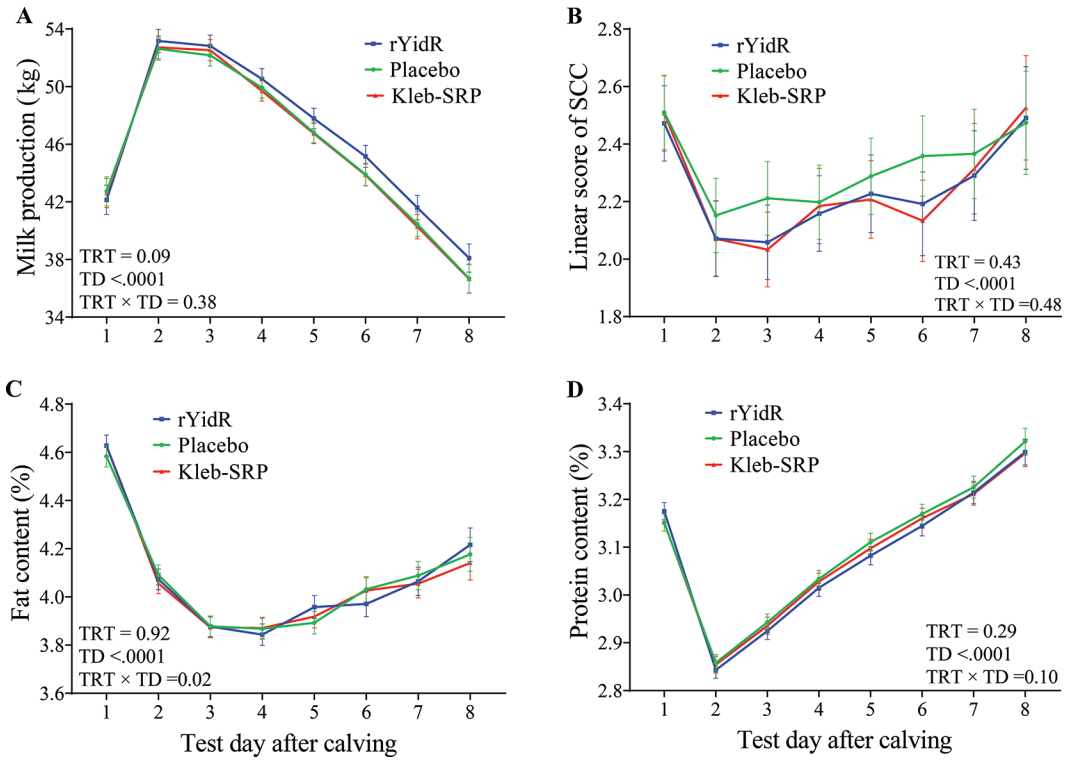

Figure 2. Milk production (A), linear score of SCC (B), milk fat content (C), and milk protein content (D) for the first 8 mo after calving of cows subcutaneously injected with one of the following treatments: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and aluminum hydroxide adjuvant (placebo). The vaccinations (2 mL/injection) were administered at the dry-off for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second inoculation was given to all animals 21 ± 3 d after the first inoculation. TRT = fixed effect of treatment; TD = fixed effect of test date; TRT × TP = fixed effect of interaction between treatment and test date. Results are presented as LSM with 95% confidence limits.

clinical signs and BW loss than the control group. Another interesting finding from the latter study was the high level of amino acid sequence homology observed when the rYidR subunit sequence was aligned against the homologous sequences of other coliform species, including the *E. coli* O157:H7 strain (pairwise identity of 79%). High-level similarities between *K. pneumoniae* and *E. coli* were also observed in terms of predicted antigenic regions and secondary structures (Rodrigues et al., 2020). Therefore, it is plausible that the positive effects of the rYidR vaccine against *E. coli* observed in the present study may be due to the host immune response acquired after vaccination. The lowest risk of culling and the lowest losses in milk production of cows that experienced *E. coli* CM supports this possibility. Outcomes from the murine study along with the results of the present study emphasize the protective effect of the rYidR vaccine against the main coliforms causing IMI in dairy cows.

Unexpectedly, the use of Kleb-SRP did not reduce the risk of CM caused by *Klebsiella* spp. compared with the placebo group, and no difference between groups was observed in the prevention of *E. coli* CM. The Kleb-SRP vaccine is commercially available and is labeled to be used for prevention of IMI caused by *Klebsiella* spp. In contrast to our findings, a recent study reported reductions of 76.9% and 47.5% in the risk of *Klebsiella* spp. mastitis and total coliform mastitis, respectively (Gorden et al., 2018). Contradictory results between studies may be partially explained by the differences in management practices and immunization protocols used in each herd, risk of exposure to *Klebsiella* spp., different strains and species of *Klebsiella* between the 2 study populations, and the number of enrolled animals. In the study by Gorden et al. (2018), the risk of mastitis was analyzed by using conditional logistic regression of pair-stratified data (i.e., exposed or not exposed to the vaccine), and no additional predictors were added to the models. In our study, we analyzed the data using multivariate logistical regression models. In addition, we enrolled 3,107 cows in our study, whereas Gorden et al. (2018) analyzed only 67 pairs of cows (immunized vs. control). Further research conducted in different farms, with different housing systems and management

practices, is required to elucidate the efficacy of Kleb-SRP against *Klebsiella* mastitis.

Differences of target antigens between the vaccines may also have affected the protective outcomes against *Klebsiella* spp. between studies. Kleb-SRP is composed of a *K. pneumoniae* siderophore receptor and porin protein, which is associated with the iron-acquisition system of bacteria (Miethke and Marahiel, 2007). On the other hand, the *yidR* gene is present within *Klebsiella* and *E. coli* genera and was selected from dozens of genes by *in-silico* analysis followed by murine studies (Yang et al., 2019; Rodrigues et al., 2020). Nevertheless, differences of immunogenic responses between vaccines across the herds may be expected due to the high heterogeneity of *Klebsiella* spp. causing CM and specific virulence features associated with the species.

Although rYidR vaccination significantly decreased the risk of *Klebsiella* CM, enrolled animals that were infected with *Klebsiella* spp. were at a similar risk of mortality or culling following the mastitis event. This result may be a consequence of the higher pathogenicity and virulence of *Klebsiella* spp. among the coliforms causing mastitis in dairy cows (Schukken et al., 2012). On the other hand, rYidR-immunized cows that were diagnosed with *E. coli* CM had approximately 55% reduction in the probability of death or culling compared with cows in the placebo and Kleb-SRP groups. Although the severity score of CM was not assessed in our study, the reduced risk of mortality or culling in the rYidR group may be associated with attenuation of the acute inflammatory response triggered by the immune system after *E. coli* CM. This finding is corroborated by the fact that rYidR-immunized cows affected with *E. coli* CM had the lowest losses in milk production following the mastitis when compared with the placebo and Kleb-SRP vaccine groups. Impressively, cows in the rYidR group produced 5.8 and 6.4 kg more milk on time point 2 (average of milk production of third and fourth test days following *E. coli* CM) compared with the placebo and Kleb-SRP groups, respectively. In

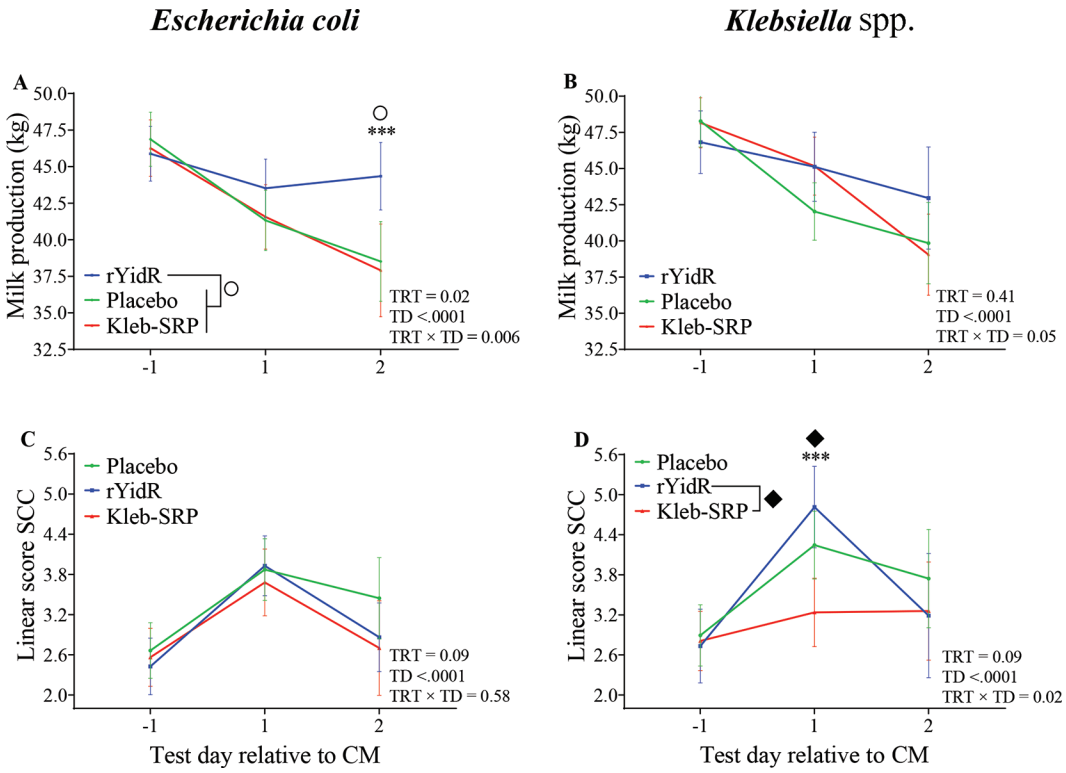

Figure 3. Milk production and linear score of SCC relative to the occurrence of clinical mastitis (CM) caused by *Escherichia coli* (A and C) and *Klebsiella* spp. (B and D). Time point −1 was generated based on the average of 2 test dates performed before CM diagnosis, whereas the time points 1 and 2 corresponded to the averages of the first and second, and third and fourth test dates after CM diagnosis, respectively. Vaccination protocols at enrollment: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and aluminum hydroxide adjuvant (placebo). The vaccinations (2 mL/injection) were administered at the dry-off for cows, and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second inoculation was given to all animals 21 ± 3 d after the first inoculation. TRT = fixed effect of treatment; TD = fixed effect of test date; TRT × TP = fixed effect of interaction between treatment and test date. Results are presented as LSM with 95% confidence limits. Empty circles and full diamonds represent differences between groups at $P \leq 0.05$ according to the figure legends.

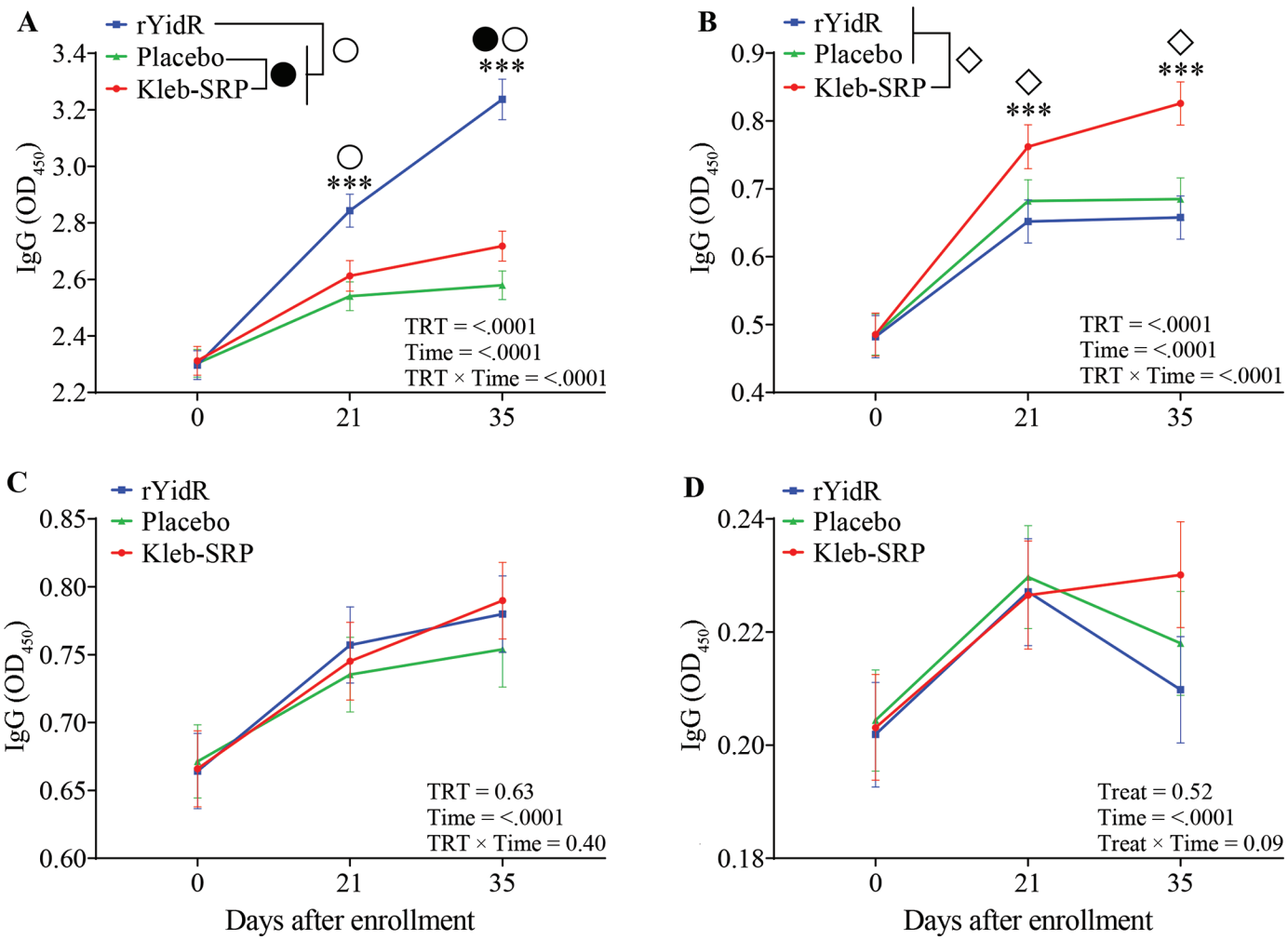

Figure 4. Effect of vaccination on ELISA-detected serum IgG against rYidR protein (A), Kleb-SRP vaccine (B), *Escherichia coli* (C), and *Klebsiella pneumoniae* (D). At enrollment, all animals were assigned to 1 of 3 vaccination protocols: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and aluminum hydroxide adjuvant (placebo). The vaccinations (2 mL/injection) were administered at the dry-off for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second inoculation was given to all animals 21 ± 3 d after the first inoculation. TRT = fixed effect of treatment; Time = fixed effect of the sample collection day; TRT × Time = fixed effect of interaction between treatment and day of blood sample collection. Results are presented as LSM with 95% confidence limits. Empty and full circles and empty diamonds represent differences between groups at $P \leq 0.05$ according to the figure legends.

addition, milk production tended to be higher for cows in the rYidR group regardless of the mastitis-causing pathogen in comparison to cows in the other groups (Figure 5A). Hence, it is plausible that vaccination with rYidR may be associated with less extensive damage of secretory tissue after CM caused by coliforms, but investigating this potential pathology was beyond the scope of the study.

In addition to its beneficial effects on mastitis observed in our study, the rYidR vaccine showed exciting results in terms of reproductive performance. Overall (regardless of parity), the conception risk at the first AI tended to be higher in the rYidR group than in the placebo group. Moreover, the conception risk at the first AI in primiparous cows immunized with rYidR was significantly higher than that observed in the placebo group, and no difference was observed between the placebo and Kleb-SRP groups. These results may be partially explained by the lowest incidence of retained placenta in rYidR-immunized cows, even though only a tendency of effect was observed. After stratification of the data by parity, primiparous cows in the rYidR group had the lowest risk of retained placenta (3.5%) compared with placebo (6.1%) and Kleb-SRP (6.0%) groups, although no effect of treatments was observed (data not shown). The unexpected effect of rYidR vaccine on the risk of retained placenta may be associated with an increase of neutrophil function in fetal cotyle-

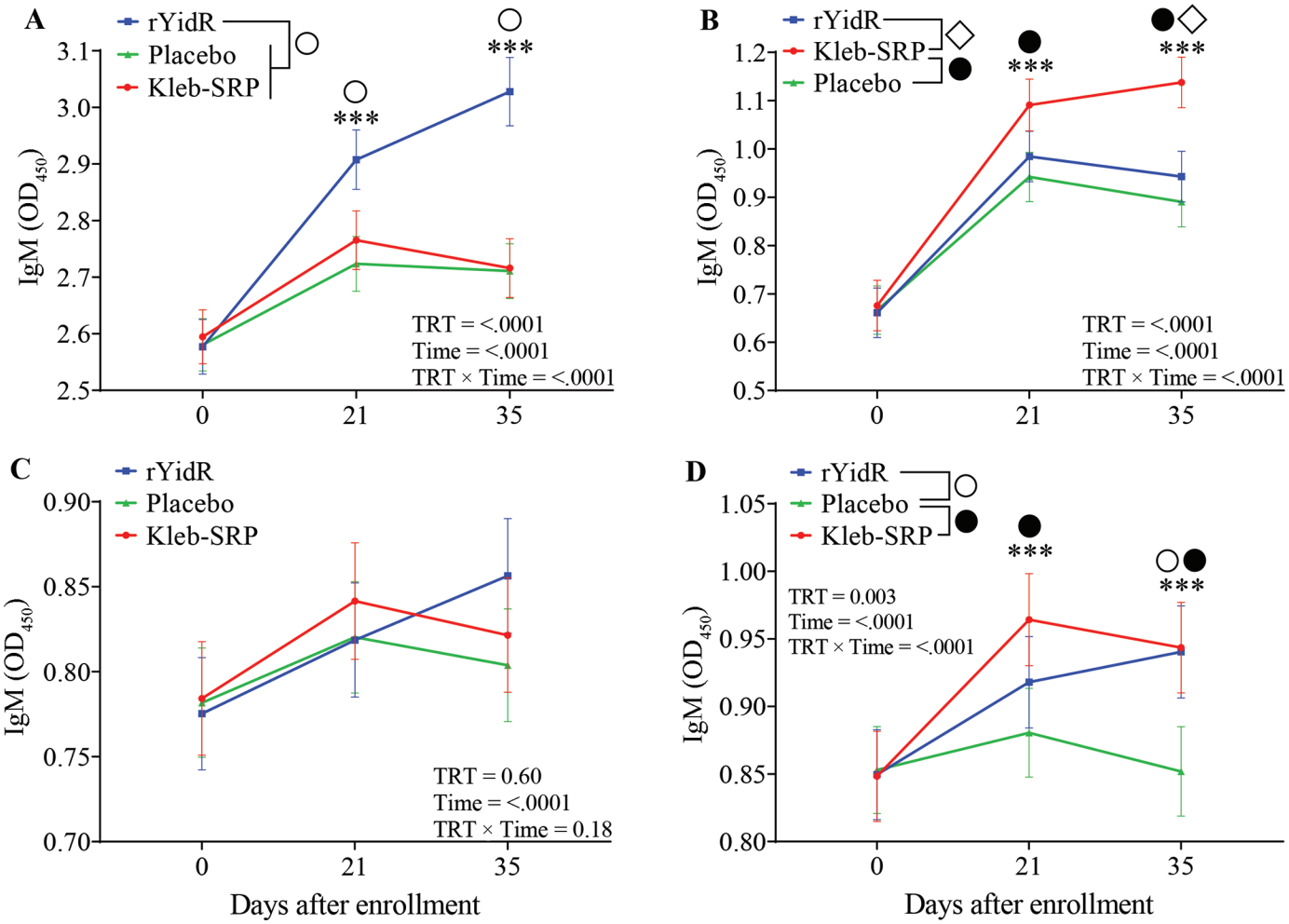

Figure 5. Effect of vaccination on ELISA-detected serum IgM against rYidR protein (A), Kleb-SRP vaccine (B), *Escherichia coli* (C), and *Klebsiella pneumoniae* (D). At enrollment, all animals were assigned to 1 of 3 vaccination protocols: experimental recombinant subunit vaccine containing the YidR protein (rYidR); commercial vaccine composed of *Klebsiella pneumoniae* siderophore receptors and porin protein (Kleb-SRP; KlebVax, Epitopix, Willmar, MN); and aluminum hydroxide adjuvant (placebo). The vaccinations (2 mL/injection) were administered at the dry-off for cows and at 223 ± 3 d of pregnancy for pre-fresh heifers. A second inoculation was given to all animals 21 ± 3 d after the first inoculation. TRT = fixed effect of treatment; Time = fixed effect of the sample collection day; TRT × Time = fixed effect of interaction between treatment and day of blood sample collection. Results are presented as LSM with 95% confidence limits. Empty and full circles and empty diamonds represent differences between groups at $P \leq 0.05$ according to the figure legends.

don tissue after calving (Kimura et al., 2002). However, for this hypothesis to be valid, the rYidR vaccine would have to trigger a general immunomodulatory response, but such an evaluation was not an objective of our study.

In addition, the highest conception risk at the first AI in rYIDR-immunized primiparous cows may have been affected by the lowest risk of CM in this group. A recent study reported that cows diagnosed with CM caused by gram-negative pathogens had 52.8% decrease of pregnancy after the first AI than cows that did not have CM (Dalanezi et al., 2020). Reduction in reproductive performance after CM have been associated with the release of inflammatory components into the blood stream, which could lead to luteolysis and embryo death in cows (Hansen et al., 2004). Moreover, assuming that pathogenic *E. coli* bacteria play a role in the development of metritis in dairy cows (Bicalho et al., 2010; Bicalho et al., 2012; Machado et al., 2014), the rYidR vaccine may had reduced the deleterious effect of pathogenic *E. coli* population in the uterus, although the treatments did not affect the metritis occurrence in our study. Nevertheless, it is worth stating that it was farm personnel, instead of researchers, who were responsible for the diagnosis of both retained placenta and metritis in our study, and potential diagnostic errors were not controlled. Only puerperal metritis cases that were accompanied by fever (i.e., puerperal metri-

tis; Sheldon et al., 2006) were treated and recorded into the farm management software and, therefore, assessed in our study. Cows that had purulent vaginal discharge but were not systemically ill were not accounted for the assessment of metritis, which may have underrepresented the frequency of this uterine disease in our population. This speculation may be conceivable, since the rYidR antigen used in the present study is highly homologous to the YidR protein ubiquitously found in *E. coli* strains (Rodrigues et al., 2020). Further studies with a more specific design are needed to better understand the effect of the rYidR vaccine on uterine diseases and reproduction indices in dairy cows.

As expected, cows in the Kleb-SRP and rYidR groups had higher serum IgG and IgM titers compared with placebo group when the respective vaccine antigens were used for the ELISA immunoassay. In addition, antibody induction (anti-*Kleb. pneumonie* IgM) was higher in vaccinated cows (Kleb-SRP and rYidR) compared with cows in the placebo group. Those results suggest that the immune system of vaccinated cows was able to recognize the antigens used for immunization and trigger an antibody response against *Kleb. pneumoniae*, although the vaccination with Kleb-SRP did not reduce the risk of *Klebsiella* CM in our study. In addition, our results showed an increase in the serum antibodies in the placebo group, especially from enrollment to d 21 of the study. That result may be associated with a serological immune response against the J5 antigens, since the cows enrolled in the study were all injected with that vaccine during lactation and precalving periods. Because J5 is a bacterin-based vaccine that is produced using an *E. coli* strain, the vaccination may have triggered a nonspecific immune response in all cows, including those in the placebo group. That speculation may be reasonable, since previous studies described that immunization with J5 bacterin produced cross-reactive antibodies with different coliforms other than *E. coli* (Dosogne et al., 2002; Chaiyotwittayakun et al., 2004).

As a consequence of sustained efforts to reduce the prevalence of contagious mastitis-causing pathogens, opportunistic bacteria such as coliforms are now among the main causes of CM in dairy herds (Ruegg, 2017). Cows affected with CM caused by *E. coli* and *Klebsiella* spp. experience dramatic declines in milk production, exhibit increased SCC, and are more likely to die or be culled during lactation (Hertl et al., 2010; Schukken et al., 2012). Furthermore, in herds that do not perform selective treatment of CM, coliforms remain a significant reason for antimicrobial use. Results from our study show that the rYidR vaccine may be a promising alternative for the control of major coliform causing mastitis in dairy herds. Further studies evaluating the efficacy of rYidR in different herds and without use of the J5 vaccine are encouraged to confirm and better understand the efficacy of the novel vaccine in prevention of coliform mastitis and diseases affecting the reproductive tract of dairy cows. In addition, investigations assessing different doses of rYidR should be performed to define the most effective immunization protocol against mastitis-causing coliforms in dairy cows. Furthermore, quantitative approaches for estimation of the magnitude of serum antibody responses after immunization with rYidR should be carried out to increase the comparability of results between studies and between vaccination protocols.

CONCLUSIONS

The rYidR vaccine reduced the risk of CM caused by *Klebsiella* spp. and the risk of culling or death of cows after CM caused by *E. coli*. In addition, rYidR-immunized cows tended to produce more milk (regardless of parity), had the lowest loss in milk production after *E. coli* mastitis, and had the highest conception rate at first service compared with cows in the Kleb-SRP and placebo groups. Based on our study population, vaccination with Kleb-SRP was not protective against CM caused by *Klebsiella* spp. and *E. coli*.

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