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Original Article

Evaluation of the humoral immune response to a multicomponent recombinant vaccine against *S. aureus* in healthy pregnant heifers

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ABSTRACT

Staphylococcus aureus is a worldwide pathogen that causes mastitis in dairy herds. Shortcomings in control programs have encouraged the development of vaccines against this pathogen. This study evaluated the vaccine candidate VacR, which included recombinant *S. aureus* protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt), formulated with a novel immune-stimulating complex. Comparisons were made between healthy pregnant heifers that received either VacR ($n = 8$; VacR group) or phosphate buffered saline (PBS) plus adjuvant (control group) SC in the supramammary lymph node area on days 45 and 15 before the expected calving date. Blood and foremilk samples were collected from 7 to 60 days post-calving.

After calving, heifers in the VacR group produced higher total IgG (IgG_{total}) titers against each component, in both serum (rBt, 3.4×10^5; rClf, 3.1×10^5; rFnBP, 2.3×10^5) and milk (rBt, 2.6×10^4; rClf, 1.3×10^4; rFnBP, 1.1×10^4), than control heifers ($P < 0.0001$). There were increased concentrations of IgG_1 and IgG_2 in VacR group ($P < 0.05$), in both serum and milk. Humoral responses remained high throughout the period most susceptible to intramammary infections ($P < 0.01$). Antibodies produced against *S. aureus* rClf and rFnBP reduced bacterial adherence to fibronectin and fibrinogen by 73% and 67%, respectively ($P < 0.001$). Milk antibodies against these adhesins inhibited *S. aureus* invasion of a mammary epithelial cell line (MAC-T), resulting in 15.7% of bacteria internalized ($P < 0.0001$). There was an approximately 6-fold reduction in the hemolysis titer for the native hemolysin in the VacR group compared to the control group ($P < 0.0001$) and a significantly increase in the proportion of positive neutrophils (VacR, 29.7%; PBS, 13.1%) and the mean fluorescent index (VacR, 217.4; PBS, 152.6; $P < 0.01$) in the VacR group. The results suggest that VacR is a valuable vaccine candidate against *S. aureus* infections, and merits further field trials and experimental challenges.

Introduction

Staphylococcus aureus is one of the most prevalent mastitis pathogens in dairy herds worldwide (Zecconi et al., 2006; Persson et al., 2011). Classical control programs based on antibiotic therapy and milking-time hygiene (Dodd and Jackson, 1971) have failed to eliminate bacterial infections; therefore, research efforts have been focused on vaccine development as a complementary control measure (Barkema et al., 2006). There is strong evidence that *S. aureus* vaccines using recombinant DNA technology yield the best results (Middleton, 2008; Anderson et al., 2012; Pozzi et al., 2012); however, only few studies have evaluated these vaccines in cattle (Pereira et al., 2011). In a previous study, our group evaluated a formulation which included some recombinant antigens in combination with a *S. aureus* lysate (Lys + Rec group; Camussone et al., 2014a). Incorporation of the recombinant molecules contributed to a more robust immune response than lysate alone (Lys group); however, the protection given by recombinant proteins alone was not assessed. The aim of this study was to evaluate a recombinant vaccine that included only recombinant antigens and an appropriate adjuvant for this type of formulation. It is understood that subunit vaccines are poor immunogens when

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administered alone; hence, the adjuvant is a key component in the formulation (Nascimento and Leite, 2012; Mohan et al., 2013). Ideal adjuvants for subunit vaccines upgrade antigen immunogenicity and serve as delivery systems to elicit optimal immune responses that cannot be achieved with traditional adjuvants (Singh et al., 2006; Mohan et al., 2013). Some recently developed immune-stimulating complexes combine antigens and adjuvant in the same nanoparticle, triggering balanced humoral/cellular immune responses in many different animal models (Morein et al., 2004; Pearse and Drane, 2005; Sun et al., 2009). Previous work by our group evaluated the performance of a new generation adjuvant (ISCOMATRIX, Isconova) as part of a project to develop a staphylococcal vaccine. When combined with different antigens from *S. aureus*, the adjuvant was effective in stimulating robust immune responses in the blood and milk of heifers (Camussone et al., 2013, 2014a,b).

In this controlled study, a recombinant multicomponent vaccine composed of three crucial virulence factors of *S. aureus* and formulated with the same novel adjuvant was prepared. Clumping factor A (rClf) and fibronectin binding protein A (rFnBP) were selected because of their roles in mammary gland invasion, whereas beta hemolysin (rBt) was used because it causes injury to the host when bacteria colonize tissues (Camussone and Calvinho, 2013; Scali et al., 2015). The potential of this new vaccine candidate to induce a robust humoral immune response was assessed.

Materials and methods

Vaccine formulation

E. coli BL21 (DE3) clones expressing *S. aureus* proteins rBt, rClf or rFnBP had been used for a previous study by our research group (Camussone et al., 2014a) and were available in our laboratory. Data about the cloning process, protein sequences and antigen purification are reported in Camussone et al. (2014a).

The multicomponent vaccine was composed of 200 μg/dose of each of three recombinant proteins (sterilized by filtration) and formulated with 2 mg/dose of adjuvant (ISCOMATRIX, Isconova). The candidate immunogen was named VacR. Heifers sham-inoculated with phosphate buffered saline (PBS) and adjuvant were used as control group (PBS). Vaccine sterility was tested by plating 100 μL of the formulation on blood agar plates in duplicate, and incubating at 37 °C for 48 h.

Animals and sampling

Sixteen healthy pregnant Holstein heifers were selected from the dairy herd at the Instituto Nacional de Tecnología Agropecuaria (INTA) Rafaela Experiment Station and randomly divided by draw in two groups of eight heifers each. Two doses of either VacR (VacR group; $n = 8$) or PBS plus adjuvant (control group; $n = 8$) were administered to heifers SC in the supramammary lymph node area on days 45 and 15 before the expected calving date (days −5 and −15, respectively). On day −15, udders were clinically examined by palpation; samples of pre-partum mammary secretion were collected using standard procedures (Oliver et al., 2004) and cultured to test for the presence of *S. aureus*. Foremilk samples were collected weekly between days 7 and 30, and on day 60 after calving. Samples were acidified by adding acetic acid (1 drop/mL) and then neutralized before centrifugation ($300 \times g$ for 15 min), as described in previous experiments (Camussone et al., 2013, 2014a). Whey samples were stored at −20 °C until used.

Blood samples were collected from the coccygeal vein before each vaccination or PBS plus adjuvant administration and on days 7, 14, 21, 30 and 60 after calving (+7, +14, +21, +30 and +60). All the procedures followed the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999) and were approved by the Committee of Animal Ethics and Security of the Facultad de Ciencias Veterinarias, UNL (Protocol No. 85/11, 30th May 2011).

Antibody determination

Indirect ELISA was used to measure specific antibodies against rBt, rClf and rFnBP in serum and whey samples from each heifer. To characterize the kinetics of total IgG (IgG_{total}) production, antibody concentrations (expressed as optical densities; ODs), were determined at days −45, −15, +7, +15, +30 and +60. Briefly, flat-bottomed 96-well microtiter plates (Greiner Bio-One International) were coated overnight with rBt, rClf or rFnBP (0.5 μg/well) mixed with carbonate buffer (pH = 9.6) at 4 °C. Serum samples were titrated to a 1:8000 concentration while whey samples were titrated to a 1:2000 concentration, following optimization in preliminary experiments. Mouse anti-bovine IgG_{total}/horseradish peroxidase (HRP; Sigma–Aldrich) was used as a secondary antibody. The assay was visualized by adding 3,3′,5,5′-tetramethylbenzidine as a substrate; after 10 min, H_2SO_4 (0.5N; Invitrogen) was added to stop the colorimetric reaction. The OD was measured at 450 nm on a plate reader (BioTek, ELx808).

IgG_{total} titers were determined following the procedure described above, but using 2-fold serially diluted serum or whey samples, taken on day +7. Titers were determined by a previously described linear regression method (Crowther, 2008). IgG_1 and IgG_2 subclasses were also measured by ELISA in serum and whey samples extracted on day +7. Mouse anti-bovine IgG_1/HRP (Sigma–Aldrich) or a mouse anti-bovine IgG_2 (Sigma–Aldrich) were applied as secondary antibodies, as appropriate, followed by a rabbit anti-mouse IgG/HRP as marker reagent (Jackson Immunoresearch).

Inhibition of the hemolytic activity of the native beta toxin (nBt)

The nBt protein was partially purified from a culture supernatant of a *S. aureus* strain isolated from a bovine mastitis case and characterized to produce only nBt in vitro (Calvinho and Dodd, 1994). Hemolytic activity against sheep erythgrocytes was assessed by pre-incubating the nBt with 2-fold serially diluted serum samples from vaccinated heifers. The hemolytic titer was the last serum dilution which caused lysis of erythrocytes. In this experiment, hemolytic titers were defined as the highest dilution that caused complete hemolysis.

Blocking assays

Fibronectin (Fn) protein was acquired commercially (Invitrogen) and fibrinogen (Fg) was purified from bovine plasma (Ismail, 2012). Blocking assays were conducted as previously described (Camussone et al., 2014a). A suspension of 1×10^9 CFU/mL of *S. aureus* Reynolds strain was pre-incubated with either anti-rClf or anti-rFnBP antibodies and purified by affinity from serum samples; these samples were collected from vaccinated heifers 7 days post-calving. Mouse sera with specificity for *S. aureus* Reynolds and a rabbit anti-mouse IgG/HRP conjugate (Sigma–Aldrich) were used in this assay. Results presented here are expressed as binding percentage, with 100% binding indicating bacteria that were not pre-incubated with serum samples.

Internalization assay

The established bovine mammary epithelial cell line (MAC-T; Huynh et al., 1991) was used. MAC-T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), insulin (5 μg/mL), hydrocortisone (1 μg/mL), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL; Sigma–Aldrich). The bacterial internalization assay was performed as previously described (Camussone et al., 2014a). *S. aureus* cells (Reynolds strain) were opsonized with whey samples diluted 1/10, obtained from each heifer on day 7 post-calving. Each assay was run in triplicate. Data are expressed as percentage of internalization compared to the control group (100%).

Opsonophagocytic assays

Bovine polymorphonuclear cells (PMN) were obtained from healthy cattle, as previously described (Siemsen et al., 2007). For the opsonophagocytosis assays, fluorescein-labeled cells from *S. aureus* Reynolds (1×10^8 CFU/mL in Hanks balanced salt solution, HBSS) were incubated with heat inactivated sera obtained from each heifer on day 7 post-calving, as described in detail previously (Camussone et al., 2014a). Fluorescence intensity was read by flow cytometry (FACSCanto II, BD Biosciences) and data was analyzed using WinMDI software. Results are expressed as the proportion of PMN containing ingested bacteria (% positive neutrophils) and mean fluorescence intensity (MFI; Zetterlund et al., 1998).

Statistical analysis

Statistical analyses were performed using GraphPad Instat 4.0 software (GraphPad). Differences beteween the vaccine and control groups were analyzed using the non-parametric Mann–Whitney test. The Kruskall–Wallis non-parametric test was used when several groups compared, followed by pairwise comparison using Mann–Whitney U-tests.

Results

Adverse reactions were not detected in either group and all calves were born healthy. IgG_{total} kinetics are represented in Fig. 1A and the curves for rBt, rClf and rFnBP were similar in shape. Each dose of VacR increased IgG_{total} and maximum concentrations were achieved on day 7 post-calving. IgG_{total} was significantly higher in the VacR group than the control group until the end of the experiment ($P < 0.05$). Serum IgG_{total} titers obtained on day +7 in the VacR group were statistically higher than those in the control

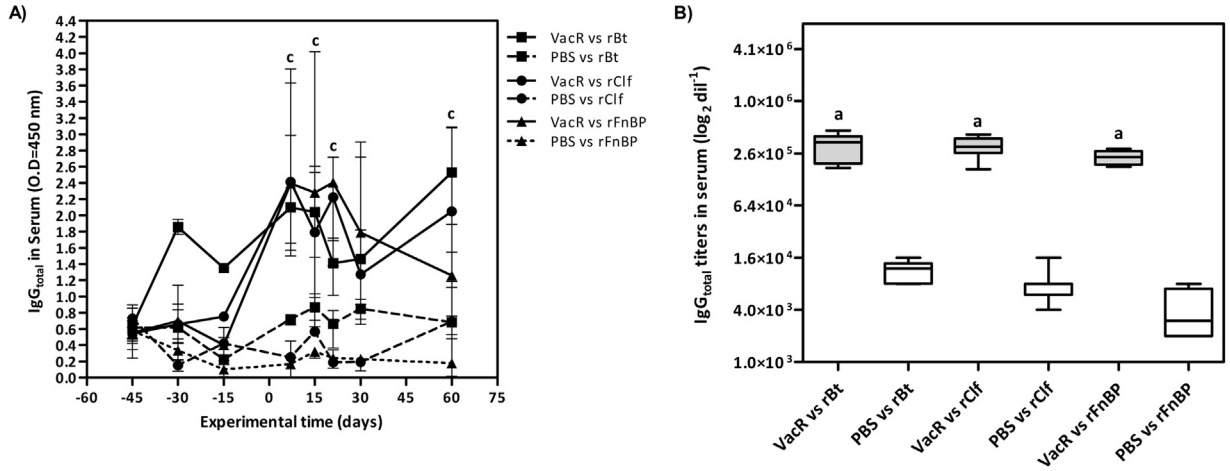

Fig. 1. Total IgG (IgG$_{total}$) response in serum. (A) Kinetics of antibody production against the *S. aureus* protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt) in serum from heifers vaccinated with the vaccine candidate (VacR) or phosphate bufferred saline (PBS), expressed as optical densities (ODs) throughout experimental days. (B) IgG$_{total}$ titers were determined for each antigen in serum samples taken on day 7 post-calving. Median (min and max) values obtained for each vaccine component are shown in the graphs. Letters indicate significant differences between VacR and PBS groups for each antigen (a, $P < 0.0001$; c, $P < 0.01$).

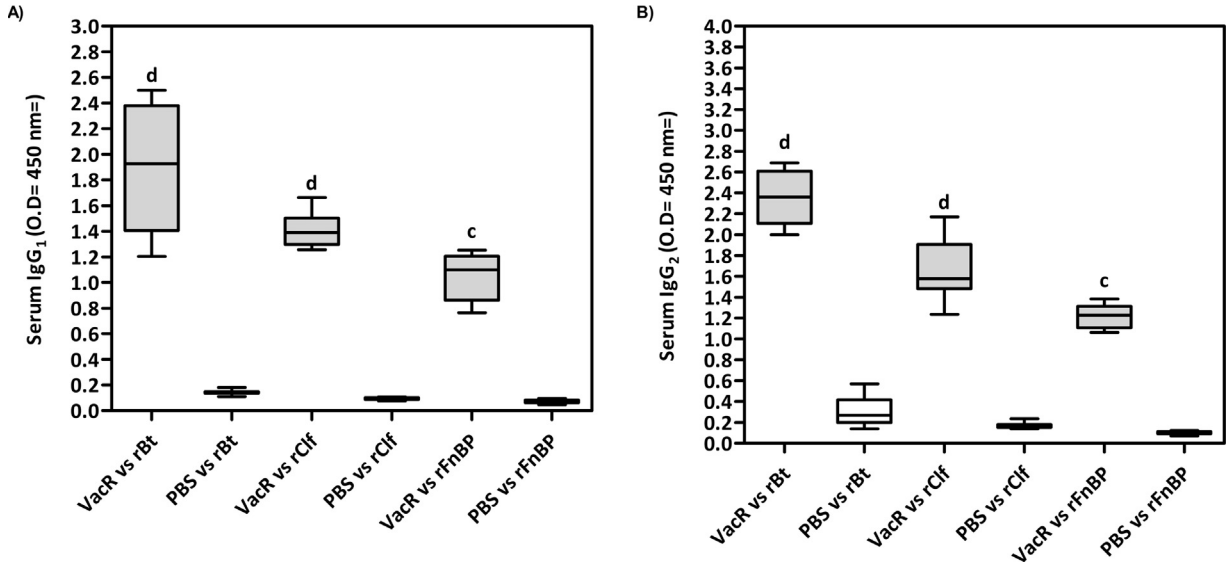

Fig. 2. IgG subclases in serum. IgG$_1$ (A) and IgG$_2$ (B) titers raised against the *S. aureus* protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt) were determined for the vaccine candidate (VacR) and phosphate bufferred saline (PBS) groups, in serum samples taken on day 7 post-calving. Median (min and max) are shown. Letters indicate significant differences between VacR and PBS groups for each antigen (a, $P < 0.000$; b, $P < 0.001$).

group ($P < 0.0001$; Fig. 1B). In the VacR group, antibody profiles demonstrated robust IgG$_1$ and IgG$_2$ subclass responses against each of the three vaccine components; these were not detected in control group heifers ($P < 0.05$; Fig. 2).

Humoral responses in milk were similar for rBt, rClf and rFnBP (Fig. 3A). Maximal IgG$_{total}$ ODs were detected on day +7 and decreased progressively over time. IgG$_{total}$ ODs remained significantly higher in the VacR group than in the control group ($P < 0.05$) during the first month after calving (until day +30). At day +7, antibody titers against each antigen were higher in the VacR group than the control group ($P < 0.05$; Fig. 3B). Milk IgG profiles demonstrated relatively high concentrations of the specific IgG$_1$ subclass and low but significant higher concentrations of IgG$_2$ in whey samples in the VacR group compared with control group ($P < 0.0001$and $P < 0.05$, respectively; Fig. 4).

The capacity of the humoral response to inhibit the vaccine targets was assessed (Table 1). Antibodies against rBt were tested using hemolytic activity assays which employed the native toxin; the hemolytic titer of nBt was 2.1×10^6. Pre-incubation of nBt with serum samples from heifers in the VacR group inhibited the hemolytic activity of the protein, resulting in a median titer of 228.3, which was significantly lower than for the control group ($P < 0.0001$).

The functionality of antibodies against the recombinant adhesins was evaluated using blocking assays. Pre-incubation of *S. aureus* cells with specific antibodies against rClf or rFnBP reduced Fg and Fn adherence by 73% and 67%, respectively, compared with the control group ($P < 0.001$). The functional capacity of adhesion-specific milk antibodies was evaluated using MAC-T internalization assays (Fig. 5). When *S. aureus* was pre-incubated with whey samples from heifers in the VacR group, the ability of bacteria to adhere to and internalize into bovine epithelial cells was significantly reduced by more than 80% compared to the control group ($P < 0.0001$).

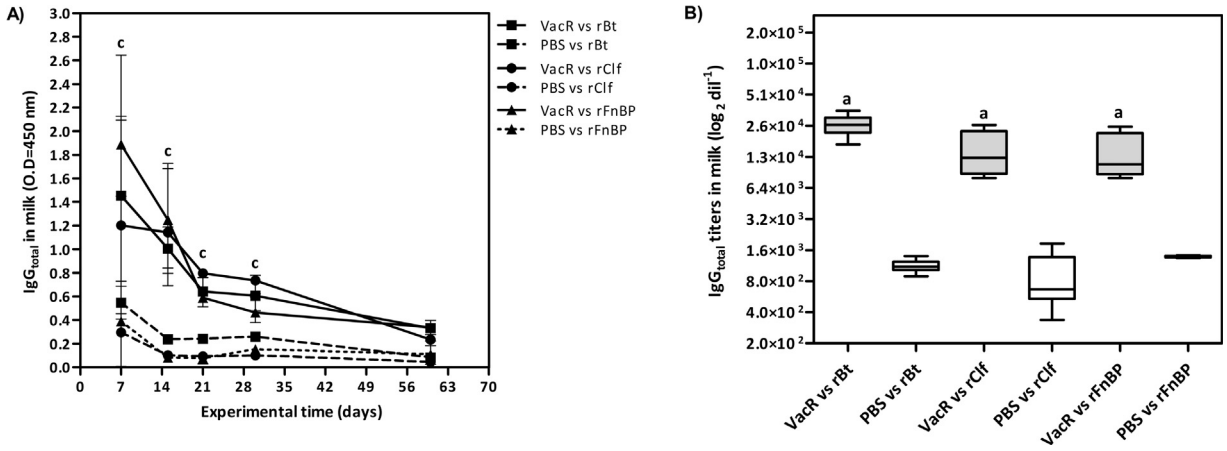

Fig. 3. Total IgG (IgG$_{total}$) response in milk. (A) Kinetics of antibody production obtained against the *S. aureus* proteins protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt) in whey samples from heifers vaccinated with the vaccine candidate (VacR) or phosphate bufferred saline (PBS), expressed as optical densities (ODs) throughout experimental days. (B) IgG$_{total}$ titers were determined for each antigen in serum samples taken on day 7 post-calving. Median (minimum and maximum) values obtained for each vaccine component are shown in the graphs. Letters indicate significant differences between VacR and PBS groups for each antigen (a, $P < 0.0001$; c, $P < 0.01$).

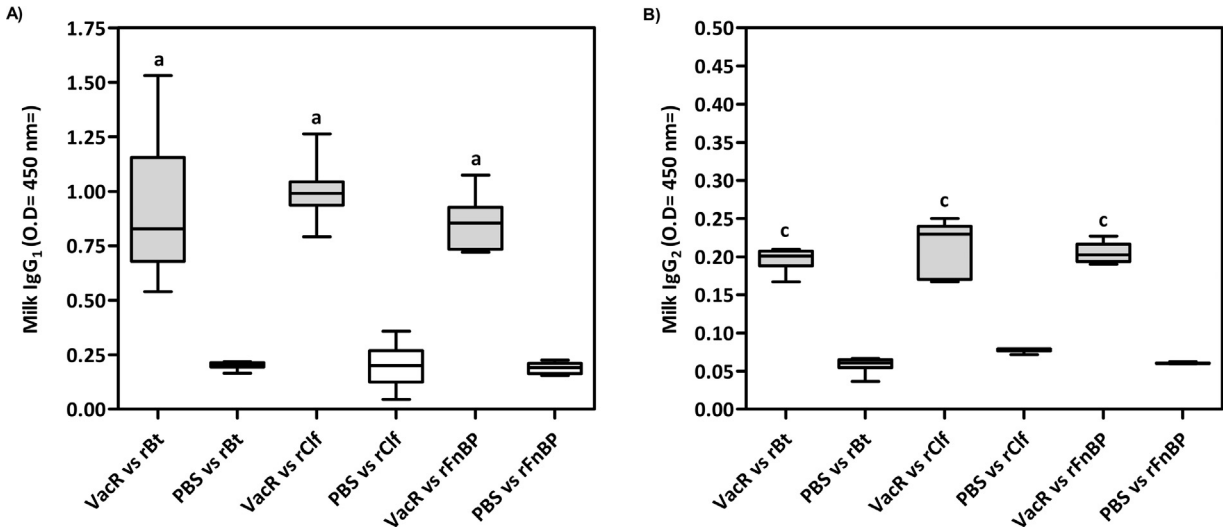

Fig. 4. IgG subclasses in milk. IgG$_1$ (A) and IgG$_2$ (B) titers raised against the *S. aureus* proteins protein clumping factor A (rClf), fibronectin binding protein A (rFnBP) and hemolysin beta (rBt) were determined for the vaccine candidate (VacR) and PBS groups in whey samples taken on day 7 post-calving. Median (minimum and maximum) are shown. Letters indicate significant differences between VacR and phosphate bufferred saline (PBS) groups for each antigen (a, $P < 0.0001$; c, $P < 0.01$).

Table 1
Functional assays. Serum antibodies obtained at day 7 post-calving from the vaccine candidate (VacR) and phosphate bufferred saline (PBS) groups were evaluated in vitro for their capability to block their specific targets. The median (min–max) are shown.

	VacR	PBS	Assay control
Hemolysis titer	228.3 (125.3–334.7)[a]	1522 (1349–1737)[c]	2,097,153
% Fibrinogen binding	27.1 (25.6–28.9)[b]	99.1 (93.8–104.1)	100
% Fibronectin binding	32.5 (27.7–44.6)[b]	98.1 (94.4–99.6)	100

[a] $P < 0.0001$ compared with assay control.
[b] $P < 0.001$ compared with assay control.
[c] $P < 0.01$ compared with assay control.

Finally, the contribution of the humoral response to *S. aureus* opsonophagocytosis was tested in vitro (Fig. 6). Pre-incubation of bacteria with sera from heifers in the VacR group produced a significant increase in phagocytic neutrophils and in the number of ingested bacteria compared with the control group ($P < 0.05$).

Discussion

Commercially available vaccine formulations to help prevent bovine *S. aureus* intramammary infections are hampered by the diversity of infecting strains and complex pathogenic mechanisms used by these microorganism (Boerhout et al., 2015). Current immunogens are traditional formulations based on whole-cell bacteria, bacterial lysates or purified bacterial components, and many are reviewed in other articles (Middleton, 2008; Pereira et al., 2011). Published evidence suggests that antibodies raised against native staphylococcal antigens lack neutralization abilities and that multicomponent recombinant vaccines produce the best results, because they induce humoral responses associated with highly functional antibodies (Middleton, 2008; Anderson et al., 2012; Pozzi et al., 2012). Additionally, recombinant vaccines are widely preferred because of their known composition, relative standardization, safety and low cost (Nascimento and Leite, 2012). However, they have poor immunogenicity and require suitable adjuvants to produce adequate humoral responses. Accordingly,

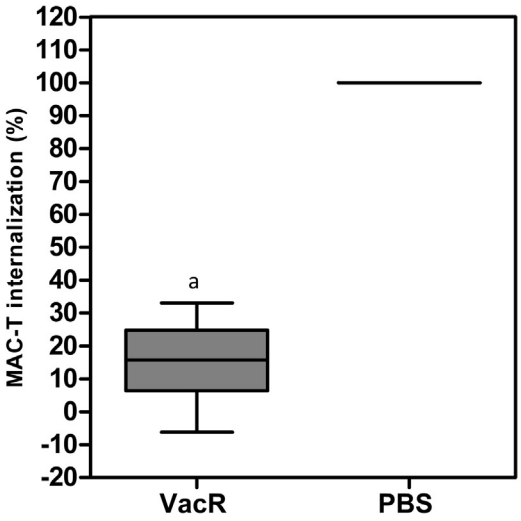

Fig. 5. Bacterial internalization into mammary epithelial cells (MAC-T). *S. aureus* cells were pre-opsonized with whey samples from the vaccine candidate (VacR) and phosphate bufferred saline (PBS) groups taken on day 7 after calving and incubated with MAC-T cells. Median percentages (min and max) are shown. The letter indicates statistically significant differences from PBS group (a, $P < 0.0001$).

the goal of this work was to evaluate the antibody response in pregnant heifers to a multicomponent vaccine which included recombinant antigens and was formulated with a new-generation adjuvant (O'Hagan and Fox, 2015). The multicomponent formulation VacR was highly immunogenic in heifers, triggering a high humoral response during the first month after calving, the period when mammary glands are highly susceptible to bacterial infection (Shafer-Weaver et al., 1999; Sordillo, 2005). Pre-existing antibodies against the three components of the vaccine were detected in all heifers, possibly due to high natural exposure to *S. aureus* in the experimental herd. Other heifer studies have demonstrated pre-immune specific antibodies against staphylococcal antigens (Shkreta et al., 2004; Boerhout et al., 2016; Mella et al., 2017). The antibody curves obtained in our study clearly showed that pre-existing antibodies remained relatively stable in control heifers throughout the experimental period, while antibody concentrations in the VacR group increased after vaccination. Peak IgG_{total} titers at day +7 confirmed that each of the three antigens used evoked high antibody concentrations and that vaccination produced both IgG_1 and IgG_2 subclasses. Robust humoral responses are highly desirable, since antibody transport occurs from the peripheral blood to the mammary gland, where antibodies contribute to local immunity (Boerhout et al., 2015).

Milk IgG_{total} curves were shaped as expected for milk from healthy bovine mammary glands, showing decreasing concentrations of antibodies associated with continuous milk secretion over the lactation period (Burton and Erskine, 2003). Our IgG_{total} and antibody profile results suggest that VacR vaccination stimulated production of the main humoral components required for udder protection. Both IgG subclasses play important roles as infection progresses. IgG_1 is passively transported from blood to mammary gland and binds preferentially to Fc gamma receptors on local macrophages, contributing to the immune defense in the early stages of mammary infection (Paape et al., 2000; Burton and Erskine, 2003). IgG_2 is mainly translocated through the blood-udder barrier linked to Fc gamma receptors on neutrophils, and is crucial for the elimination of bacteria by phagocytosis in the later stages of infection (Paape et al., 2002; Sordillo and Streicher, 2002; Burton and Erskine, 2003). Studies evaluating whole-cell bacteria-based immunogens did not find specific IgG_2 in milk after vaccination (Barrio et al., 2003; Luby et al., 2007; Camussone et al., 2014a). However, recombinant antigens have stimulated detectable concentrations of vaccine-induced IgG_2 in milk (Shkreta et al., 2004; Nour El-Din et al., 2006; Boerhout et al., 2015). In this study, we also detected increased concentrations of vaccine-specific IgG_2 in whey samples from the VacR group, which provides further suggests that recombinant proteins could be efficacious as immunogens. The presence of IgG_1 and IgG_2 in the healthy udder could indicate immune surveillance ready for potential infections.

In vitro assays were performed to evaluate the functional properties of antibodies in the blood and milk. There is a dearth of published information about the functional properties of bovine antibodies stimulated by vaccination against *S. aureus*. Previous works evaluating native beta toxin as an immunogen (purified from a staphylococcal strain) intended to generate neutralizing antibodies reported discouraging results in heifers (Nordhaug et al., 1994; Watson, 1992). Similarly, other authors have reported that native ClfA failed to generate functional antibodies, and that only recombinant proteins evoked protective immune responses (Higgins et al., 2006; Hawkins et al., 2012). Conversely, results from studies of recombinant vaccines against *S. aureus* have demonstrated their ability to stimulate the production of neutralizing antibodies (Middleton, 2008; Anderson et al., 2012; Pozzi et al., 2012; Camussone et al., 2014a). In our previous work, we reported

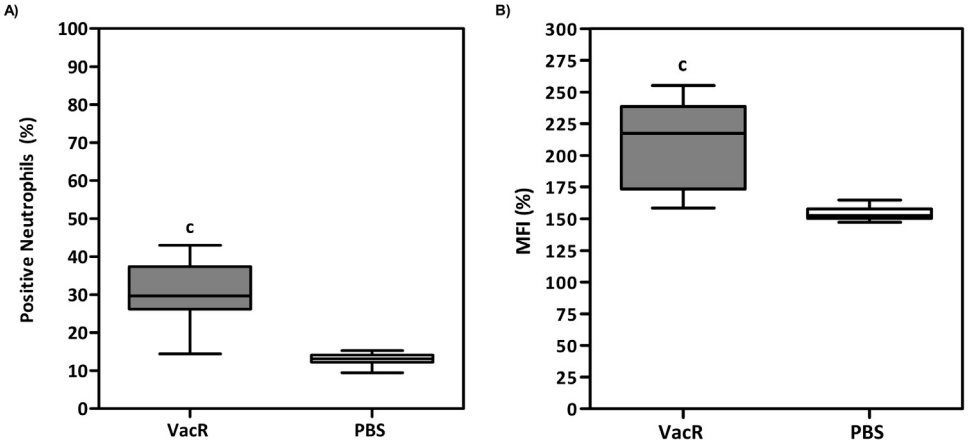

Fig. 6. Opsonophagocytosis assay. *S. aureus* cells were pre-opsonized with serum samples from the vaccine candidate (VacR) and phosphate bufferred saline (PBS) groups taken on day 7 after calving and incubated with bovine polymorphonuclears cells (PMN). Median percentages (min and max) are shown. The letter indicates statistically significant differences from the PBS group (d, $P < 0.05$).

that the incorporation of rBt, rClf and rFnBP in a *S. aureus* lysate vaccine (Lys + rec) contributed to the production of antibodies that significantly inhibited the functions of their specific targets when compared with the lysate alone (Lys) or a control group (Camussone et al., 2014a). However, we did not test the potential immunogenicity of recombinant proteins alone. In the present study, in vitro assays were performed to investigate the functionality of the humoral response evoked by VacR, a formulation which includes only recombinant antigen. Hemolytic activity tests and blocking assays showed that the antibodies produced were capable of effectively recognizing and neutralizing the specific target expressed by *S. aureus*. Neutralization of beta toxin could reduce cell damage caused by the pathogen, and blocking the main bacterial adhesins might prevent tissue invasion. Moreover, ClfA from *S. aureus* impaired phagocytosis through interaction with complement factor I, resulting in increased C3b degradation (Hair et al., 2010). Earlier studies which evaluated recombinant ClfA as an immunogen in animal models have reported increased phagocytosis in vaccinated groups compared to control groups (Brouillette et al., 2002; Nour El-Din et al., 2006; Gong et al., 2010). While no antiphagocytic role has been clearly described for FnBPA, previous studies have reported significantly increased phagocytosis in animals vaccinated against this protein compared with control groups (Zhou et al., 2006;[1]). Camussone et al. (2014a) did not report significant differences in phagocytosis between heifers from *S. aureus* CP5 lysate and recombinant antigen vaccine groups. Accordingly, the usefulness of these recombinant proteins as vaccine components could be questioned. The results of this study show that VacR vaccine effectively promoted bacterial phagocytosis, the main defense mechanism against staphylococcal infections.

Since the bacterial entry site in bovine mastitis is the udder, the ability of milk directed to adhesin proteins to inhibit *S. aureus* adhesion to mammary gland tissue was investigated in vitro in MAC-T invasion assays. The few previous works evaluating recombinant vaccines in heifers have reported that antibodies directed against adhesins reduced *S. aureus* adherence to MAC-T cells (Shkreta et al., 2004; Nour El-Din et al., 2006). In our study, invasion assays demonstrated that specific antibodies against rFnBP and rClf in milk effectively inhibited bacterial internalization into MAC-T cells.

Conclusions

Vaccination of pregnant heifers with VacR evoked a robust humoral response against each vaccine component in blood and milk during the first month after calving, which is a period of increased susceptibility to acquire intramammary infections. The functional assays reported here strongly indicate that the immune response could impair bacterial invasion and host damage mechanisms and promote bacterial elimination by phagocytosis. Our study provides good evidence of the immunogenic potency of recombinant antigens from *S. aureus* and suggest that VacR is a possible vaccine candidate. These results are promising and encourage us to continue experimental challenges to determine the effectiveness of VacR to help prevent bovine *S. aureus* mastitis. However, since *S. aureus* infections are multifactorial, the incorporation of other molecules to VacR formulation to extend protection should be considered.

[1] See: Morein, B., Lövgren-Bengtsson, K., Ranlund, K., Fromgren, B., Ekstrom, J., Baschunan, C.C., 2011. Vaccine composition comprising a fibronectin binding protein or a fibronectin binding peptide. US 8,007,806 B2. http://www.freepatentsonline.com/8007806.html. (Accessed 8 March 2018).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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